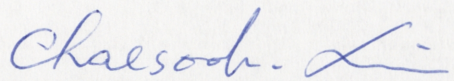
Interaction of the proteins which regulate MHC class II-mediated antigen presentation by antigen presenting cells.

Maria Chaesook Yang

A Thesis Submitted for the Degree of Master of Science of
The Australian National University
September, 1998

Statement

I have performed all of the experiments described in this thesis. The work presented here has been an independent intellectual enterprise, except for acceptable practical input from my supervisor and other interested persons.



Maria Chaesook Yang

Division of Biochemistry and Molecular Biology

John Curtin School of Medical Research

The Australian National University

September 1998

Acknowledgments

I wish to thank my supervisor, Dr. Peter Milburn, and my advisors, Dr. Arno Mulbucher and Dr. Anand Gautam, for the excellent supervision and advice they have given me throughout this work. Special thanks go to Jan Bateman, Pam Bleakley and Phil Bunyan for their support and encouragement to start and finish this course successfully. I also would like to thank Simon Prasad, Sarah Weenink and Aparna Bhatnager, who have provided me with advice and an environment of encouragement in the lab.

I am very grateful to the photography staff, Stuart Butterworth, Karen Edwards, Marc Fenning and Julie Macklin, to the computer staff, Michael Smith and Sue Henderson, and to the staff of the FACS facility, Geoff Osborn and Sabine Gruninger for their constant help with the new technology.

My husband Hongsuk, and my two lovely daughters, Anne and Jenny, have been a constant source of support during this time and I certainly would not have gotten far without them. Finally, I would like to extend my special thanks to my mother for her spiritual support in all my endeavors.

Abstract

In antigen presenting cells, a non-classical major histocompatibility (MHC) class II molecule, HLA-DM, plays an important role in MHC class II antigen presentation by acting as a molecular chaperone which facilitates peptide binding to classical MHC class II molecules. In the endoplasmic reticulum (ER), MHC class II molecules associate with invariant chain (Ii) and they are co-transported to antigen processing compartments where internalized and processed antigenic peptides are present. Efficient delivery of antigenic peptide to the cell surface by class II molecules is enabled by DM which catalyzes the exchange of class II-associated Ii peptides (CLIP) from the peptide binding groove for antigen-derived peptides. The complete role of DM and its interactions with other molecules in the MHC class II pathway remains to be established.

The results presented in this thesis demonstrate intracellular association of H2-M (the murine analogue of HLA-DM) with both the mouse MHC class II molecule, I-A^d, and 31kDa Ii immediately after synthesis in the ER and, further, a co-operative role in the degradative processing of Ii. Molecules participating in the I-A^d class II antigen presentation process were extracted from cell lysates by immumoprecipitation using various solution conditions and then the presence of co-precipitants was identified by autoradiography and Western analysis. Methodology was developed to screen for co-precipitation with solution conditions varying in detergent and pH. Cell lysis with different detergents indicated that the intracellular association of H2-M is weak compared to that of the other proteins (I-A^d or Ii). Also, the pH dependence of co-immunoprecipitation revealed that complex stability was sensitive to pH. Analysis of pulse-chase experiments by double-immunoprecipitation revealed that H2-M was associated with I-A^d and intact Ii-31 immediately after synthesis in the ER and with I-A^d and successively

smaller Ii fragments at later time points. The association of H2-M with Ii-31 at pH7 was stable enough not to be dissociated in the strongly chaotropic detergent, NP40. The analysis of an I-A^d deleted cell line, M12.C3, demonstrated that the H2-M/Ii association may occur directly, independent of the presence of I-A^d, and that I-A^d/H2-M co-operation may be required for the efficient degradation of Ii in the normal MHC class II antigen processing pathway.

Several novel findings have been presented here concerning the association of H2-M with Ii and I-A^d, however, further studies are required to confirm the presence of an I-A^d/Ii/H2-M complex and establish its stoichiometry.

Abbreviations

2-ME	2-mercaptoethanol
APC	antigen presenting cells
ATP	adenosine triphosphate
bio	biotin conjugated
BSA	bovine serum albumin
c	complexes
CLIP	class II-associated Ii peptides
DO	HLA-DO
DMSO	dimethylsulfoxide
ECL	enhanced chemiluminescene
EDTA	ethylene diamine tetra acetate
ER	endoplasmic reticulum
FACS	Fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HLA	Human Leukocyte Antigen
HRP	horse radish peroxidase
I-A	H2-A
I-E	H2-E
Ig	immunoglobulin
Ii	invariant chain
Ii-31	31kDa Ii
$(Ii\text{-}31)_2$	dimer of Ii-31
FBS	foetal bovine serum
LIP	leupeptin-induced peptides

Ma	H2-Ma
mAb	monoclonal antibody
Mb	H2-Mb
MHC	major histocompatibility molecules
MIIC	MHC class II compartments
NHS-biotin	*N*-hydroxysuccinimido biotin reagent
Novex	Novel Experimental Technology
NP40	Nonylphenoxy polyethoxy ethanol
p	polypeptides
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-T	0.01% Tween 20 in PBS
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLIP	small leupeptin-induced peptides
TAP	Transporters associated with Antigen Processing
TCR	T cell receptors
TGN	trans-Golgi network
Tricine	Tris, *N*-tris[hydroxymethyl]methylglycine
Tris	tris[hydroxymethyl]aminomethane
Tween-20	polyoxyethylenesorbitan monolaurate

Table of contents

Chapter 1

1. Introduction

1.1. General introduction

Higher vertebrates possess two types of host defence system which protect them against infectious agents. One, which is nonspecific, pre-exists in all individuals and includes physical and chemical barriers to pathogen entry such as skin, stomach acid, mucus, ciliary respiratory lining and lysozyme. The other is the immune system which is induced by antigens and gives protection against disease.

The immune system may be classified in two parts, the innate and the adaptive immune systems, which differ in a number of important ways. Non-specific recognition of antigen is the characteristic of the innate immune system and the cells involved in innate immunity include macrophage, mast and natural killer cells. Adaptive immunity features specificity in the recognition of antigens as well as providing memory which enables rapid response to challenge by the same or related antigen in the future.

The adaptive immune response may be divided into 2 major subtypes, the humoral immune response mediated by B cells and antibody and the cellular-mediated immune response by T cells (Janeway Jr. and Travers, 1994). Professional antigen presenting cells (APC) include B cells, macrophages and dendritic cells. There are two major types of MHC molecules which enable presentation of antigenic peptides for cellular and humoral immune responses, MHC class I and II, respectively (Engelhard *et al.*, 1994). T cells recognize peptide antigens which are bound to major histocompatibility complex (MHC) molecules on the cell surface of antigen presenting cells and this recognition of antigen by T cells is specific (Wolf and Ploegh, 1995). There are two major subpopulations of peripheral T cells, $CD8^+$ T cells and $CD4^+$ T cells which

recognize antigenic peptides bound to MHC class I and class II molecules, respectively.

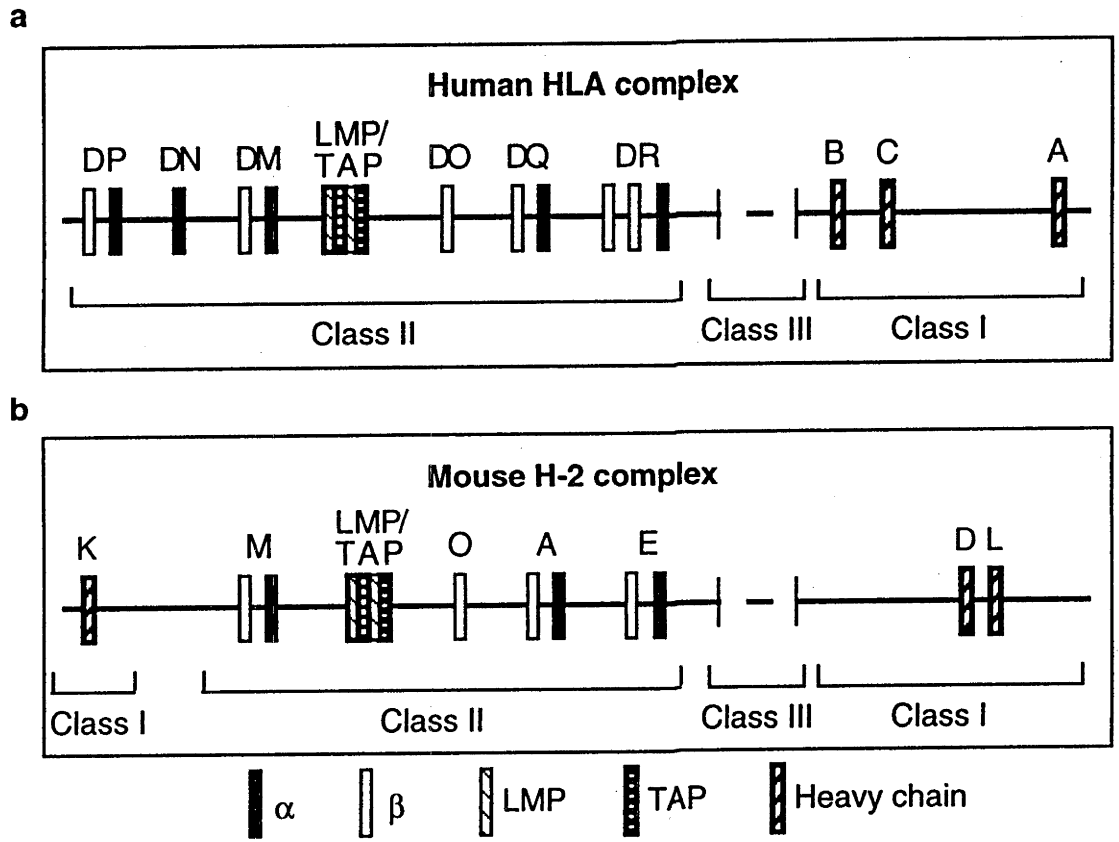
The aim of this project is to study the interaction of proteins which participate in MHC class II antigen presentation in B cells, therefore this introduction is balanced in favour of MHC class II-mediated antigen presentation by B cells.

1.2. The MHC class I and class II pathway of Ag presentation.

The MHC class I and class II molecules are encoded by a set of highly polymorphic genes, located in the MHC (Monaco, 1992; Maffei *et al.*, 1997). A schematic representation of human and mouse MHC gene organization is shown in Fig. 1.1. The MHC class I and class II molecules are cell-surface glycoproteins involved in antigen presentation and host defence. There are predominantly three class I genes expressed in both humans and mice: in humans, Human Leukocyte Antigen (HLA)-A, -B and -C and in mice, H2-D, -L and -K. In the class II region of the MHC, humans express three class II genes, HLA-DP, -DQ and -DR while mice express a maximum of two class II genes, H2-A and H2-E. MHC class I molecules present peptides derived predominantly from intracellular antigens to $CD8^+$ T cells (Townsend *et al.*, 1997) and MHC class II molecules present peptides derived predominantly from extracellular antigens to $CD4^+$ T cells (Campbell and Shastri, 1998). Therefore, peptides from different sources are presented to different T cells and, furthermore, discrimination between presentation by MHC class I and class II molecules arises from the use of different intracellular pathways (Engelhard *et al.*, 1994).

1.2.1. The structure of class I and class II MHC molecules.

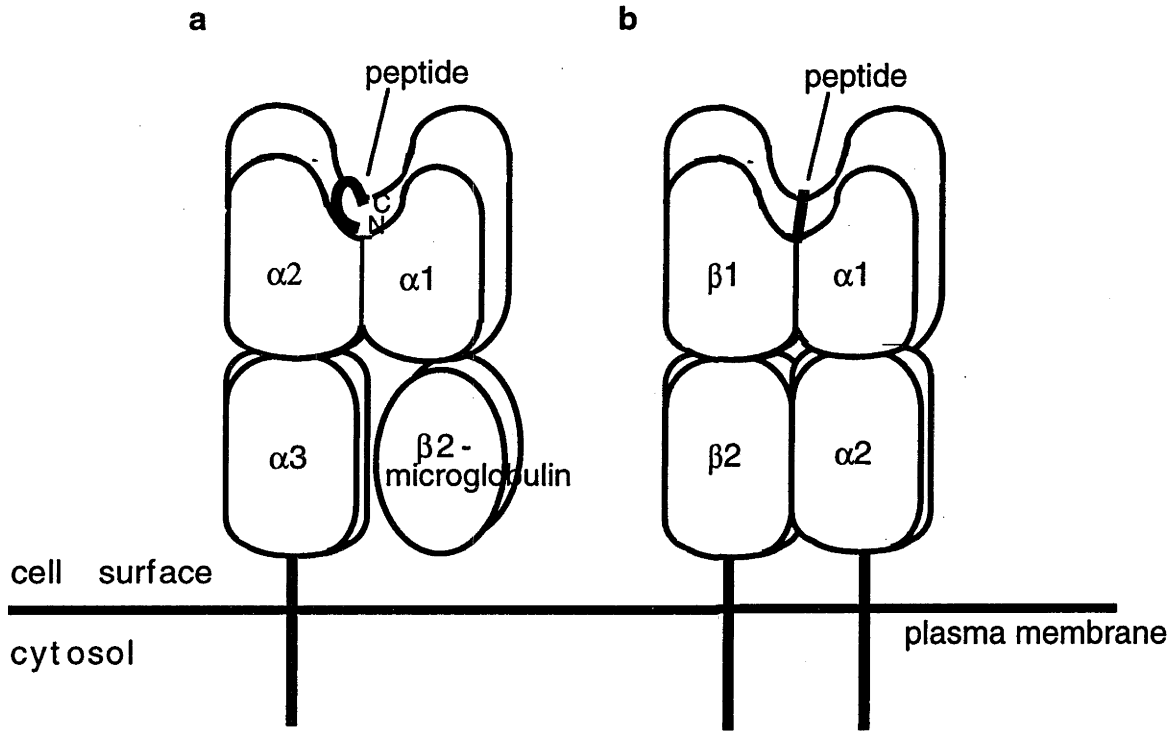
Structures of MHC class I and class II glycoproteins have been determined by X-ray crystallography (Brown *et al.*, 1993; Stern *et al.*, 1994; Murthy *et al.*, 1997;


a
Human HLA complex
DP
DN
DM
LMP/
TAP
DO
DQ
DR
B
C
A
Class II
Class III
Class I
b
Mouse H-2 complex
K
M
LMP/
TAP
O
A
E
D L
Class I
Class II
Class III
Class I
α
β
LMP
TAP
Heavy chain

Bjorkman and Mowbray, 1998). The MHC class I molecules are heterodimeric proteins which consist of a 43-45kDa molecular weight (MW) α chain and a non-covalently associated 12kDa MW subunit called β2-microglobulin. The molecules have 4 structural domains, three of which are formed from the α chain (α1, α2 and α3) and one from β2-microglobulin. The α chain spans the cell membrane but the β2-microglobulin does not (Bjorkman *et al.*, 1987).

Fig. 1.2a shows a model of a peptide binding to a MHC class I molecule. The MHC class I molecule binds peptides of defined length (usually 8-10 amino acid residues) and both ends of the peptide bound to MHC class I molecules are buried in the peptide-binding groove of the molecules (Madden *et al.*, 1992). Deep and highly conserved pockets at each end of the binding groove bind the amino and carboxyl termini of peptides through hydrogen bonds and the orientation of peptide binding is determined electrostatically (Fairchild, 1998). A deep polymorphic pocket in the middle of the binding groove provides allele-specific peptide binding (Matsumura *et al.*, 1992).

MHC class II molecules are transmembrane glycoproteins consisting of a non-covalently associated 33-35kDa MW α chain and a 25-30kDa MW β chain (Brown *et al.*, 1993). As Fig. 1.2b shows, each chain of MHC class II molecules has 2 domains (α1, α2 and β1, β2) and the peptide binding groove is constructed from one domain of each chain (α1 and β1). Peptides that bind to MHC class II molecules are longer than the peptides that bind to MHC class I molecules with no apparent restriction on peptide length, although they are usually 13-17 amino acids long (Stern *et al.*, 1994; Rudensky *et al.*, 1991). The peptide binding groove of MHC class II molecules is open at both ends and the peptides are bound in an extended conformation, projecting out of both ends of the groove (Brown *et al.*, 1993).

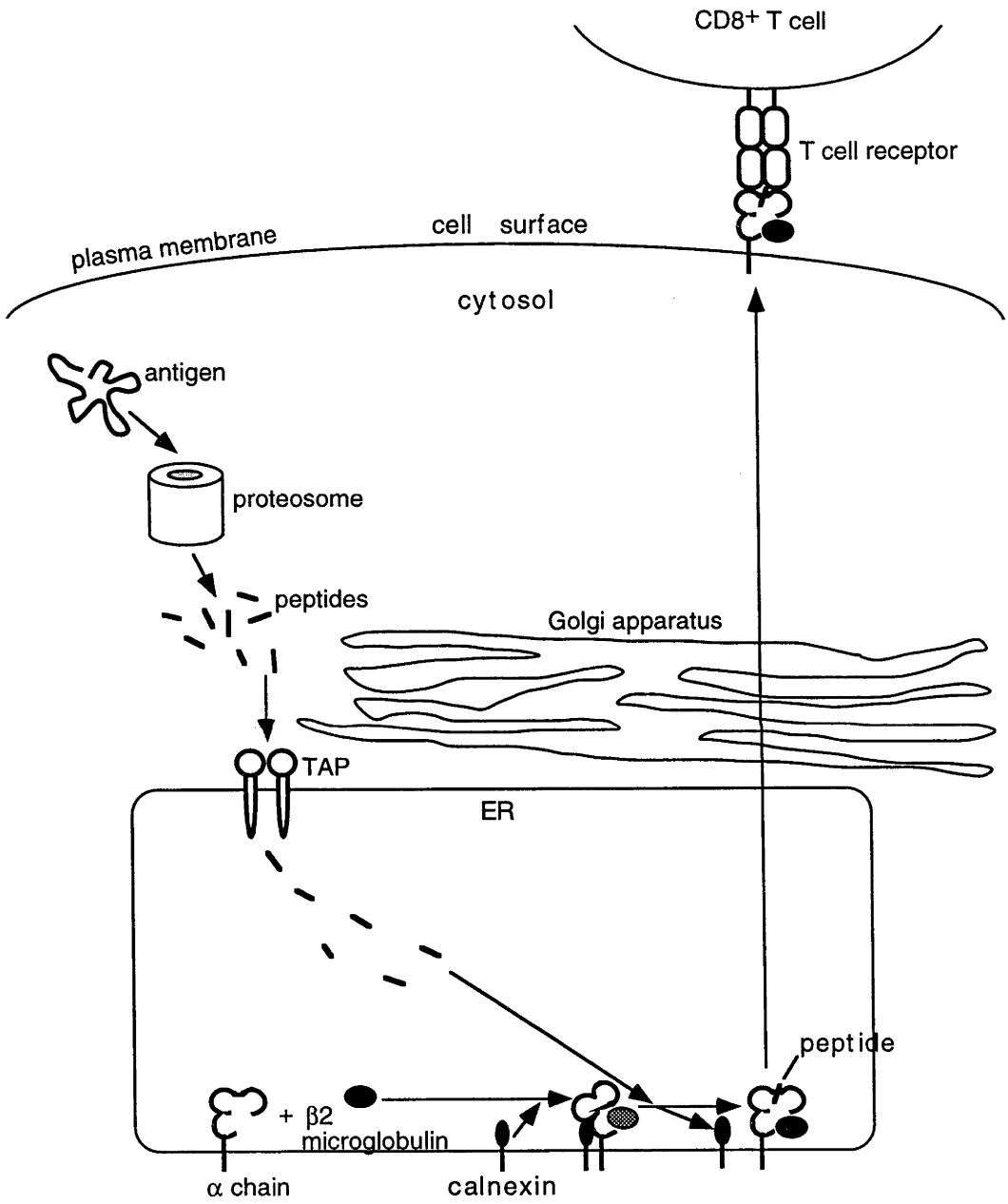

a
b
peptide
peptide
C
N
α2
α1
β1
α1
α3
β2 -
β2
α2
cell surface
cytosol
plasma membrane

1.2.2. MHC class I-mediated antigen presentation

A schematic representation of MHC class I-mediated antigen presentation is shown in Fig. 1.3. MHC class I molecules present peptides to $CD8^+$ T cells as explained above. These peptides are derived from cytosolic proteins and include both virus- and self-derived antigens (Braciale, 1992). The cytosolic proteins are partially degraded by the proteosome, a large multi-catalytic protease complex consisting of 28 subunits (Haracska and Udvardy, 1997). The genes which encode two subunits of the proteosome, LMP-2 and LMP-7, have been mapped to the MHC gene cluster and the encoded proteins have been implicated in antigen presentation (Neefjes, 1995). These peptides are then translocated into the lumen of the ER by Transporters associated with Antigen Processing (TAP). TAP are an adenosine triphosphate (ATP) dependent multi-membrane spanning heterodimer which has a preference for peptides of 8-13 amino acids in length, which is the approximate size of peptides bound by MHC class I molecules (Neefjes, 1995).

Newly synthesized and partially folded MHC class I molecules may be stabilized in the ER by calnexin, an ER-resident protein chaperone (Hughes and Cresswell, 1998), until a cognate peptide is loaded. More recently, Vigna *et al.* (1996) have suggested that an additional chaperone, invariant chain (Ii) which is intimately involved in MHC class II antigen presentation, stabilizes particular MHC class I heterodimers until a peptide is loaded and that this association may enhance class I cell surface expression.

In the ER, the partially folded MHC class I molecules are retained until they associate with a peptide to form a stable MHC class I-peptide complex which is then routed through the Golgi apparatus to the cell surface (Neefjes *et al.*, 1990). Ultimately, $CD8^+$ T cells recognise the peptide bound MHC class I molecules and become activated and elicit effector function, such as cytokine release or mediation

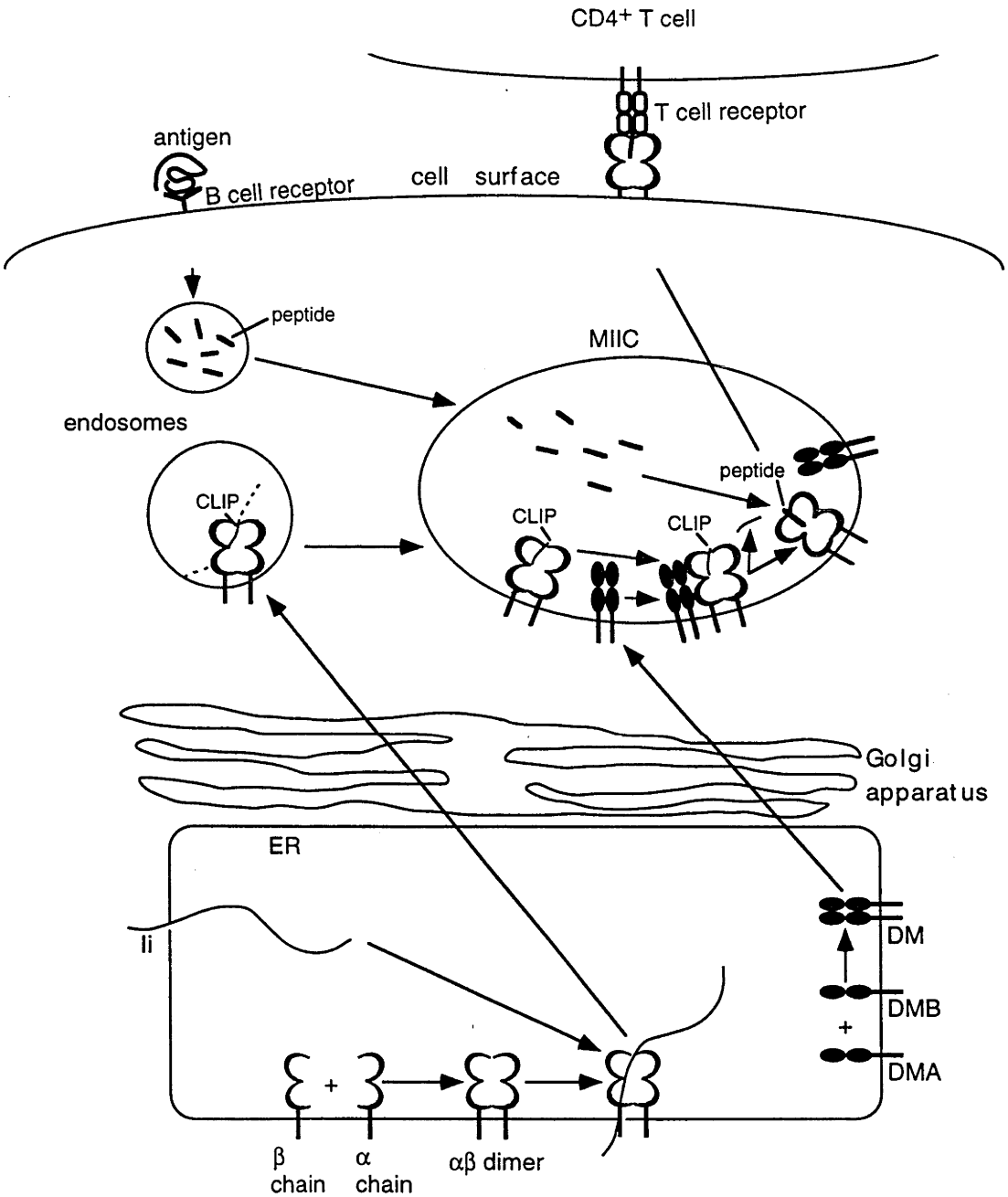

CD8+ T cell
T cell receptor
plasma membrane
cell surface
cytosol
antigen
proteosome
peptides
Golgi apparatus
TAP
ER
peptide
+ β2 microglobulin
α chain
calnexin

of cytotoxic processes *via* exocytosis of perforin and granzymes (Pinkoski *et al.*, 1998) or *via* activation of the Fas pathway on the target cells (Smyth *et al.*, 1998).

1.2.3. MHC class II-mediated antigen presentation

As Fig. 1.4 shows, extracellular antigens from potential pathogens such as bacteria or large parasites, are endocytosed and processed by the APC for presentation by MHC class II molecules to $CD4^+$ T cells. The endocytosis of antigens may be specific, being mediated by immunoglobulin (Ig) receptors expressed on B cells (Rudensky *et al.*, 1994) or may occur by non-specific mechanisms, such as fluid phase adsorptive endocytosis, phagocytotosis or autophagy (Wolf and Ploegh, 1995). The endocytosed antigen passes through early and late endosomes, where proteolysis occurs. Additionally, lysosomes process bacterial antigens for MHC class II presentation to $CD4^+$ T cells (Harding and Geuze, 1992).

After MHC class II molecules are synthesized in the ER, premature peptide binding is prevented by the association of the α/β heterodimer with a third integral membrane glycoprotein, Ii. (Riberdy *et al.*, 1992; Busch *et al.*, 1996). MHC class II/Ii complexes are then transported from the ER *via* the endocytic pathway through the Golgi apparatus where the complex undergoes glycosylation and sialylation (Lamb and Cresswell, 1992; Warmerdam *et al.*, 1996). The endocytic transport of MHC class II $\alpha\beta$/Ii complexes is mediated by targeting or retention signals in the cytoplasmic N-terminal domain of Ii (Viville *et al.*, 1993; Naujokas *et al.*, 1995; Zhong *et al.*, 1996). The endocytic pathway is commonly divided into early endosomes, late endosomes and lysosomes. In the acidic environment of endosomal compartments (Mellman *et al.*, 1986), Ii is proteolytically degraded, leaving a nested set of Ii fragments, class II-associated Ii peptides (CLIP), in the class II peptide binding groove (Rudensky *et al.*, 1991). Proteolysis of Ii occurs


CD4+ T cell
T cell receptor
antigen
B cell receptor
cell surface
peptide
endosomes
MIIC
CLIP
peptide
CLIP
CLIP
Golgi apparatus
ER
Ii
DM
DMB
+
DMA
+
β chain
α chain
αβ dimer

progressively as the pH declines and the concentration of proteases increases in the endosomal compartments (Wolf and Ploegh, 1995). Enzymes such as cathepsin B, D, E and S are involved in Ii proteolysis (Blum and Cresswell, 1988; Nguyen *et al.*, 1989; Harding *et al.*, 1991; Reyes *et al.*, 1991; Maric *et al.*, 1994; Riese *et al.*, 1996).

The MHC class II/CLIP complexes accumulate in MHC class II compartments (MIIC, Riberdy *et al.*, 1994) which are acidic (pH 4.5-5.5) multivesicular structures of late endosomes or early lysosomes which contain lysosomal protein markers, such as β-hexosaminidase (a lysosomal enzyme) and LAMP-1 (a lysosomal protein) (Peters *et al.*, 1991; Sanderson *et al.*, 1994). In the MIIC, CLIP may be dissociated from MHC class II molecules and, consequently, an antigenic peptide may be loaded in the empty groove of the MHC class II molecules (Neefjes *et al.*, 1990; Kropshofer *et al.*, 1997). Riberdy *et al.* (1994) have reported that DR3 class II molecules associated with CLIP accumulated in the MIIC in T2.DR3 cells. However, since MHC class II molecules and Ii are present throughout the endocytic pathway the actual compartment in which MHC class II/antigenic peptide complexes are formed is difficult to pinpoint (Wolf and Ploegh, 1995).

Unlike MHC class I molecules, which may reach the cell surface minutes after arrival in the trans-Golgi network (TGN), there is a 1-3 hour delay between the arrival of the class II molecules in the TGN and their subsequent deposition at the cell surface (Neefjes *et al.*, 1990). During this lag phase, the MHC class II αβ/Ii complexes are targeted to the MIIC, where CLIP are released and antigenic peptides are bound in the MHC class II molecules (Cresswell, 1994).

A third molecule, called HLA-DM in the human and H2-M in the mouse, is an accessory element for the formation of functional class II-peptide complexes. This protein is a non-polymorphic heterodimer which is structurally similar to MHC

class II molecules (Cho *et al.*, 1991; Kelly *et al.*, 1991). After being synthesized in the ER, DM is transported to the endocytic pathway and accumulates in the MIIC (Sanderson *et al.*, 1994). This localization of DM to the MIIC occurs by virtue of a tyrosine based endosomal localization signal in the cytoplasmic tail of the β chain (Copier *et al.*, 1996; Kropshofer *et al.*, 1997).

In the MIIC, DM catalyzes both CLIP removal from MHC class II molecules and peptide loading into the empty groove of MHC class II molecules (Denzin and Cresswell, 1995; Sherman *et al.*, 1996). However, the peptide exchange in the MHC class II molecules is not strictly DM-dependent and the degree of DM dependency for peptide loading differs for various MHC class II alleles (Brook *et al.*, 1994). For example, DR3-mediated antigen presentation is DM-dependent (Green *et al.*, 1995) while I-A^k class II-associated antigen presentation is not (Stebbins *et al.*, 1996). In the case of highly DM-dependent antigen presentation, direct interaction of DM with MHC class II molecules has been assumed for the catalysis of peptide exchange in the MHC class II molecules (Sanderson *et al.*, 1996). Kropshofer *et al.* (1996) have shown that DM releases self-peptides, including CLIP, bound to DR and, also, the DM-mediated peptide dissociation from DR was optimal at a pH range of 4.5-5.5, which corresponds to that of endosomal compartments where both DM and DR accumulate (Sloan *et al.*, 1995). There is much debate at present about the mechanism of peptide exchange and the nature of DM association with other molecules in the endocytic pathway.

It has been suggested that MHC class II/peptide complexes are transported to the cell surface by direct fusion of MIIC with the plasma membrane (Wubbolts *et al.*, 1996). On the cell surface, peptide bound MHC class II molecules are recognised by T cell receptors (TCR) on MHC class II-restricted $CD4^+$ T cells (Nydam *et al.*, 1998). The interaction between the MHC class II/peptide complex on the surface of the B cell (or other APC) and the TCR on $CD4^+$ T cells induces

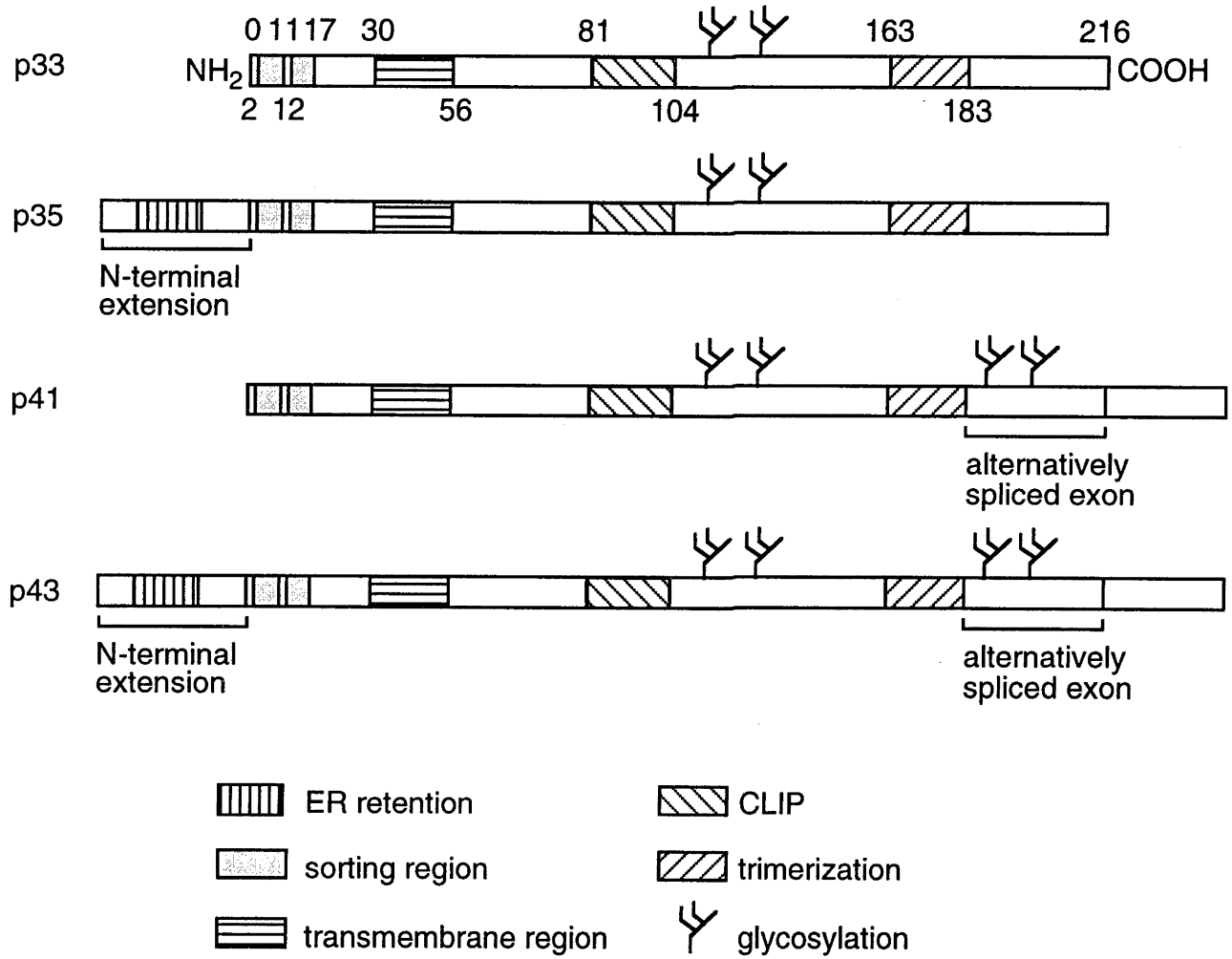
their activation. In addition to the recognition of MHC class II/peptide complexes by TCR, co-stimulatory signals, such as B7, from the APC are also required to activate the $CD4^+$ T cells (Nydam *et al.*, 1998). The activated $CD4^+$ T cells secrete a variety of cytokines with immunoregulatory properties (Torres *et al.*, 1996). Subpopulations of activated $CD4^+$ T cells in turn may stimulate B cells to differentiate and secrete antibodies (Ingvarsson *et al.*, 1995).

1.3. The role of Ii in MHC class II antigen presentation

The integral membrane glycoprotein, Ii, makes an essential contribution to the presentation of antigen by MHC class II molecules. Mice lacking Ii exhibit defects in MHC class II assembly and transport, resulting in reduced levels of cell surface expression of class II molecules, altered antigen presentation and inefficient positive selection of $CD4^+$ T cells (Viville *et al.*, 1993). However, reconstitution of these mice with Ii restores normal positive selection of $CD4^+$ T cells (Naujokas *et al.*, 1995).

1.3.1. The structure of Ii and its association with the MHC class II molecules

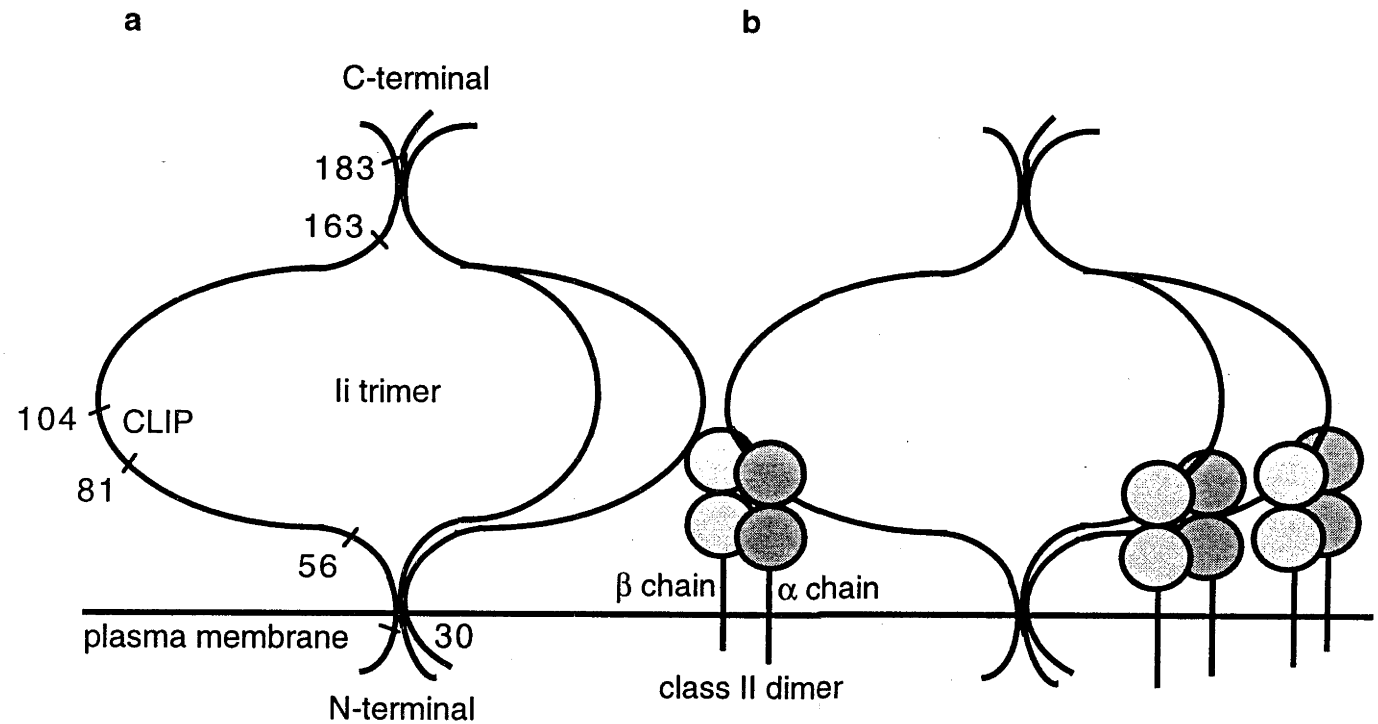
Ii is a membrane glycoprotein of 31-43kDa and, in contrast to MHC class II molecules, Ii from different haplotypes have been shown to have the same sequence such that this molecule is described as being non-polymorphic (Jones *et al.*, 1978). In addition, the Ii gene is encoded outside the MHC region (see Fig. 1.1). Ii is usually synthesized in a molar excess over class II molecules and certain cell types that do not normally express class II molecules express Ii (Cresswell, 1992). The Ii gene encodes four different molecular weight polypeptides (p): p33, p35, p41 and p43 in humans (Fig. 1.5) and p31, p33, p41 and p43 in mice (Wolf and Ploegh, 1995). The most abundant form in human B cells is p33 while, correspondingly, p31 is the most abundant form in mice. An alternative initiation


p33
0 1 11 17 30
81
163
216
NH2
COOH
2 12
56
104
183
p35
N-terminal extension
p41
alternatively spliced exon
p43
N-terminal extension
alternatively spliced exon
ER retention
sorting region
transmembrane region
CLIP
trimerization
glycosylation

of translation in humans results in p35, while both p41 and p43 are generated from alternative splicing of an additional exon to p33 and p35, respectively (Strubin *et al.*, 1986; O'Sullivan *et al.*, 1987). The mature Ii protein incorporates both N-linked and O-linked oligosaccharides (Cresswell, 1992).

Newly synthesized Ii polypeptides form homotrimeric complexes by interaction of their carboxy-terminal regions (Marks *et al.*, 1990; Lamb and Cresswell, 1992). Each of these Ii trimers bind to 3 αβ MHC class II dimers, yielding a nonameric complex, $(\alpha\beta/Ii)_3$, in the ER (Roche *et al.*, 1991; Newcomb and Cresswell, 1993; Cresswell, 1996 and Fig. 1.6). The 270kDa nonameric form of the MHC class II αβ/Ii complex leaves the ER and enters the endocytic pathway. The signals responsible for directing the MHC class II αβ/Ii complex to the endocytic pathway are between residues 2 and 17 of the cytoplasmic tail of Ii (Bakke and Dobberstein, 1990). Excess Ii also exists as trimers and those that fail to assemble with MHC class II α and β chains are retained in the ER by virtue of a retention signal in the N-terminus of p35 or p43 (Marks *et al.*, 1990; Lamb and Cresswell, 1992). Other forms of Ii, p33 and p41, may also be retained in the ER through the formation of mixed trimers containing p35 or p43.

In the acidic environment of endosomal compartments, Ii is degraded by proteases resident in these compartments (Blum and Cresswell, 1988; Maric *et al.*, 1994). Blockade with the protease inhibitor, leupeptin, leads to the accumulation of the 22kDa leupeptin-induced peptides (LIP) and the 11kDa small leupeptin-induced peptides (SLIP) which are the Ii fragments normally cleaved by one of the proteases, cathepsin B (Nguyen *et al.*, 1989; Reyes *et al.*, 1991). Both LIP and SLIP include the N-terminal region of Ii which incorporates the sorting and retention signals because they were recognized by an anti-Ii antibody derived from Ii residues 12-28 (Nguyen *et al.*, 1989). Further degradation of Ii in the endocytic


a
b
C-terminal
183
163
Ii trimer
104
CLIP
81
56
plasma membrane
30
N-terminal
β chain
α chain
class II dimer

pathway gives rise to the shortest Ii fragments, CLIP, associated with MHC class II molecules (Riese *et al.*, 1996).

Peptides eluted from MHC class II molecules include CLIP originating from residues 81-104 of Ii exon 3 which are the final fragments that remains associated with MHC class II molecules (Rudensky *et al.*, 1991; Ghosh *et al.*, 1995). An X-ray crystallographic study of a CLIP-DR3 complex has shown that the CLIP fragments bind to DR3 in a way almost identical to that in which antigenic peptides bind to MHC class II molecules (Ghosh *et al.*, 1995). In this complex, the CLIP are located directly in the peptide binding groove of DR3, where side chains of CLIP extend into pockets of the DR3 binding site and hydrogen bonds were formed between the amide groups of both CLIP and DR3.

1.3.2. The role of Ii in MHC class II antigen presentation

After MHC class II molecules are synthesized in the ER they are transported *via* the endocytic pathway to the MIIC, where an antigenic peptide is loaded in the MHC class II molecules prior to presentation to $CD4^+$ T cells. Ii makes a number of important contributions to this process by virtue of its association with the MHC class II molecules in the class II pathway. This protein chaperones MHC class II molecules, facilitating folding, precluding premature peptide binding, directing transport, stabilizing conformation and preventing aggregation (Rudensky *et al.*, 1991; Cresswell, 1992; Viville *et al.*, 1993).

The Ii markedly increases the efficiency of assembly and folding of certain MHC class II alleles. Of five haplotypes, $H\text{-}2^{b,\ d,\ k,\ u\ and\ dq1}$ in the mouse, each haplotype has two different MHC class II genes, H2-A (refer to I-A) and H2-E (refer to I-E) (see Fig. 1.1). In the case of the $H\text{-}2^b$ haplotype, the α and β subunits of $I\text{-}A^b$ assemble less efficiently into dimers in the absence of Ii and are predominantly expressed as free chains (Viville *et al.*, 1993). In contrast, $I\text{-}A^k$, I-

E^k and I-A^d dimers are assembled efficiently independent of the presence of Ii (Bikoff *et al.*, 1995).

Association of Ii with MHC class II αβ dimers prevents premature peptide binding by occluding the peptide binding groove of the MHC class II molecules in the ER. Since both MHC class I and class II molecules are capable of binding peptides in the ER, the MHC class II molecules must be prevented from becoming saturated by peptides generated from cytosolic proteins and this is largely achieved by complexation with Ii. Peptides derived from self-proteins have been eluted from purified class II molecules however the intracellular site at which they are bound was not determined (Rudensky *et al.*, 1991). It is felt that peptide binding to MHC class II molecules in the ER may be only a minor pathway of class II restricted antigen presentation (Wolf and Ploegh, 1995). Class II molecules appear to undergo a physiochemical change upon binding of cognate peptide, forming a stable and compact complex on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Stebbins *et al.*, 1996). This property has been used to determine antigenic peptide loading. In Ii deficient cells, the class II αβ dimers formed SDS-stable complexes in the ER of 1-A^k, 1-E^k, 1-E^d and HLA-DR1 transfected HeLa cells (Busch *et al.*, 1996), which demonstrates that in the absence of Ii MHC class II molecules form stable peptide adducts in the ER. Similarly, in splenocytes from Ii deficient C57BL/6 mice peptide loading in the MHC class II molecules was detected in the ER by formation of SDS-stable dimers on a SDS-PAGE gel (Shachar *et al.*, 1995). These results are a clear demonstration of the role of Ii in preventing premature peptide loading.

Ii chaperones the MHC class II αβ dimers from the ER to the endocytic pathway through the Golgi apparatus. Different isoforms of Ii are sorted to the MIIC by different transport routes and the trans-Golgi network (TGN) is the major site of signal dependent protein sorting within the secretory pathway (Cresswell,

1992; Warmerdam *et al.*, 1996). The αβ/Ii complexes are targeted to the endosomes either directly from the TGN (for MHC class II/Ii p35) or after rapid internalization from the cell surface prior to their delivery to antigen processing compartments (for MHC class II/Ii p33, Warmerdam *et al.*, 1996). The cell surface class II (HLA-DR)/Ii complexes reached endosomes by rapid internalization in .45 cells and this highly efficient endocytosis was also mediated by the cytoplasmic tail of Ii (Roche *et al.*, 1993). However, the majority of the class II/Ii complexes enter the endocytic pathway directly from the TGN (Cresswell, 1992) by virtue of localization signals in the N-terminus of Ii (see Fig. 1.5).

The Ii serves to stabilize the conformation of MHC class II molecules in the endocytic pathway until cognate peptides are bound. Cells from Ii-deficient mice of H-2^b and H-2^k haplotypes did not have the typical compact dimer conformation of I-A^b and I-A^k indicative of tight peptide binding, resulting in very poor presentation of peptide antigens (Viville *et al.*, 1993). The Ii-deficient H-2^b mice were also defective in the positive selection of $CD4^+$ T cells (Naujokas *et al.*, 1995; Wong *et al.*, 1996). Therefore, Ii may make a critical contribution to MHC class II αβ assembly, folding, transport, structural stability and, ultimately, $CD4^+$ T cell selection.

1.3.3. The role of class II-associated Ii peptides (CLIP)

The crystal structure of a HLA-DR3-CLIP complex (Ghosh *et al.*, 1995) has shown that CLIP binds in the groove of MHC class II molecules in a manner similar to an influenza virus peptide binding to DR1 (Stern *et al.*, 1994). Ghosh *et al.* (1995) have proposed also that Ii is associated with the MHC class II molecules *via* direct interaction of the CLIP region in the peptide binding groove by hydrogen bonds. This structural information is consistent with the fact that the CLIP

compete with the binding of MHC class II-specific antigenic peptides in antigen processing compartments (Riberdy *et al.*, 1992).

Although CLIP and antigenic peptides bind with the same conformation at the same binding site of the class II molecules, the class II (I-A^d)/CLIP complexes generally do not generate dimers exhibiting SDS stability (Stebbins *et al.*, 1996). This feature is very important for class II antigen presentation. If these class II/CLIP complexes were more stable than the class II/peptide complexes, it would disfavour the exchange of CLIP from the MHC class II molecules with an antigenic peptide in the binding groove of class II molecules.

Notionally, CLIP may be subdivided into 2 functional regions. The C-terminal region, residues 92-105, of the CLIP mediate competitive inhibition of peptide binding by occupying the peptide binding groove (Kropshofer *et al.*, 1995), with the conserved residues methionine 93 and methionine 99 involved in the modulation of CLIP binding affinity in the class II molecules, I-A^d and I-A^u, (Gautam *et al.*, 1995). The N-terminal region, residues 81-91, mediates the efficient release of CLIP from the MHC class II molecules (DR2) (Kropshofer *et al.*, 1995; Stumptner and Benaroch, 1997), with the conserved residues lysine 83, lysine 86 and proline 87 being responsible for making destabilizing contacts between N-terminal regions of CLIP and certain MHC class II molecules (Kropshofer *et al.*, 1995).

1.4. HLA-DM in the MHC class II pathway

During transport along the endocytic pathway, Ii is proteolytically degraded, leaving CLIP bound to MHC class II molecules. These class II αβ/CLIP complexes accumulate in the MIIC (Rudensky *et al.*, 1994) where antigenic peptides are loaded. In the MIIC a third molecule, HLA-DM, becomes involved in

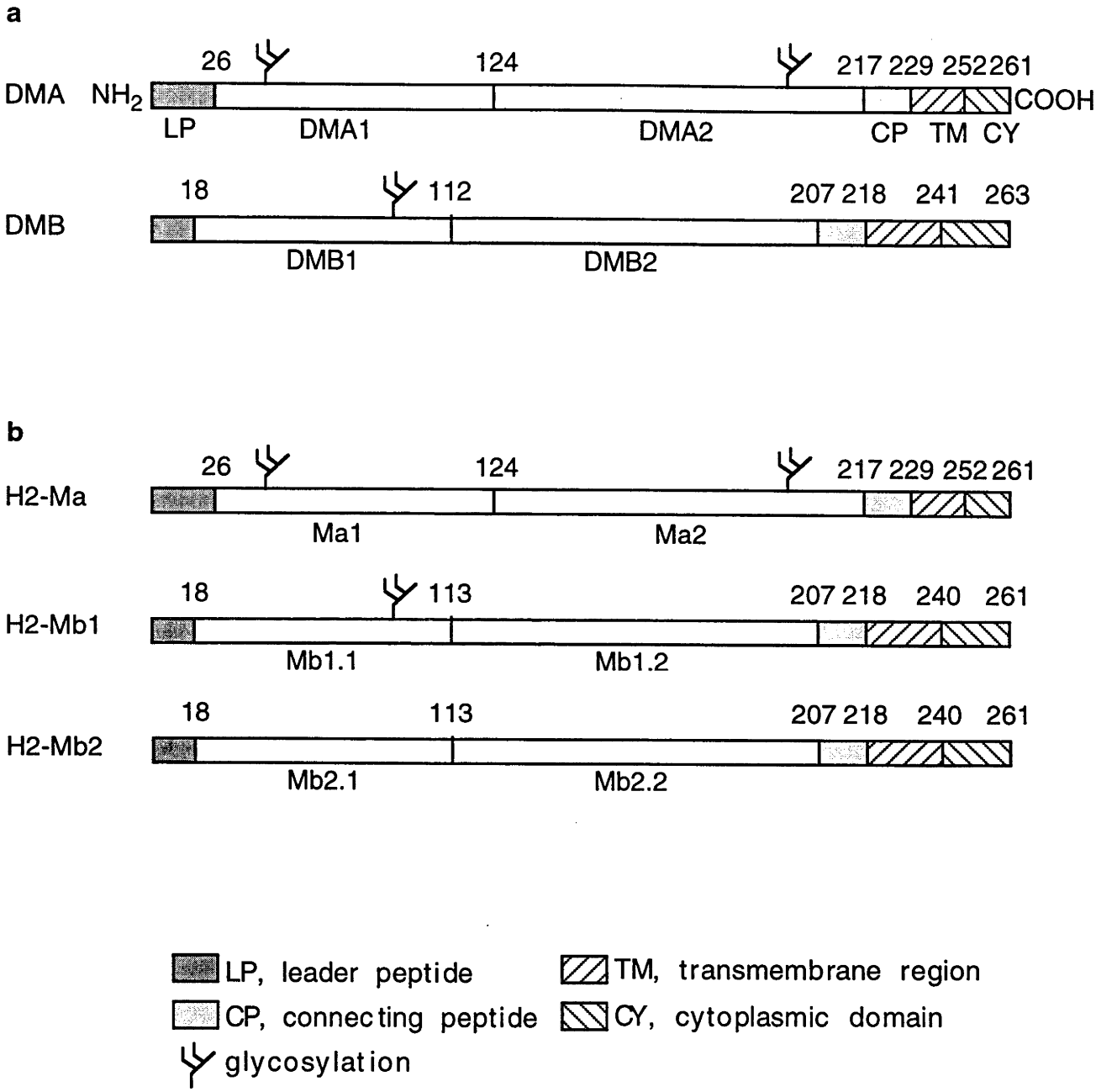
CLIP removal from the MHC class II molecules and facilitates peptide loading (Kropshofer *et al.*, 1997).

1.4.1. The structure of HLA-DM

DM is a αβ heterodimeric transmembrane glycoprotein. The schematic structural organization of human DM and mouse H2-M is shown in Fig. 1.7. The subunits of DM are encoded by 2 genes: DMA and DMB in humans and H2-Ma (Ma) and H2-Mb (Mb) in mice. These genes are located in the MHC class II region (see Fig. 1.1) and encode class II-like (non-classical) molecules (Cho *et al.*, 1991; Cho *et al.*, 1991'; Kelly *et al.*, 1991). The DM genes are constitutively expressed in B cells and, like MHC class II molecules, their expression may be upregulated by γ-interferon (INF-γ) (Cho *et al.*, 1991). In contrast to MHC class II molecules, this molecule shows limited polymorphism only (Sanderson *et al.*, 1994; Pepteraux *et al.*, 1996).

Between human and mouse, the sequence homology of DM is high (>70%, Kelly *et al.*, 1991). According to Kelly *et al.* (1991) and Cho *et al.* (1991), DMA and Ma each have 261 amino acids comprising a 33-35kDa protein with 2 N-linked glycosylations. DMB has 263 amino acids comprising a 31-33kDa protein with 1 N-linked glycosylation (Kelly *et al.*, 1991), however, there are two Mb genes: Mb1 and Mb2 (Karlsson *et al.*, 1994). According to Karlsson *et al.* (1994), the Mb2 protein sequence is quite different from the Mb1 sequence, especially, in the membrane distal domain and the site for N-linked glycosylation in Mb1 is not present in Mb2. Thus, although Mb1 and Mb2 have 261 amino acids each, Mb1 is a 31-33kDa protein with 1 N-linked glycosylation and Mb2 is a 27kDa protein with no glycosylation (Karlsson *et al.*, 1994).

DM α and DM β are predicted to be structurally similar to the class II α and β chains and thus capable of forming a heterodimer with a peptide-binding-like cleft


a
DMA
NH2
26
124
217 229 252 261
COOH
LP
DMA1
DMA2
CP
TM
CY
DMB
18
112
207 218
241
263
DMB1
DMB2
b
H2-Ma
26
124
217 229 252 261
Ma1
Ma2
H2-Mb1
18
113
207 218
240
261
Mb1.1
Mb1.2
H2-Mb2
18
113
207 218
240
261
Mb2.1
Mb2.2
LP, leader peptide
CP, connecting peptide
glycosylation
TM, transmembrane region
CY, cytoplasmic domain

(Wolf and Ploegh, 1995). However, the DM genes are different from the classical class II genes in several aspects. According to Kelly *et al.* (1991), the differences include low sequence homology between the two sets of genes, potentially more disulfide bridges in DM which would confer a more rigid structure and limited polymorphism (Sanderson *et al.*, 1994; Pepteraux *et al.*, 1996). It has been assumed that these properties may not allow tight binding of peptides in the groove of the DM molecule (Kelly *et al.*, 1991).

1.4.2. The function of HLA-DM in the class II antigen presentation pathway

HLA-DM is not expressed on the cell surface (Sanderson *et al.*, 1994), instead being retained within the endosomal system by the localization signal in the cytoplasmic tail of the β-chain (which incorporates tyrosine 230, Lindstedt *et al.*, 1995; Copier *et al.*, 1996). Denzin *et al.* (1996) have reported that incubation of DR3-CLIP complexes with DM elicited release of CLIP *in vitro*. DM accumulates in the MIIC where peptide loading occurs (Sanderson *et al.*, 1994) but is found throughout the endocytic pathway (Pierre *et al.*, 1996).

It has been shown that DM is expressed at a lower level than MHC class II molecules in .114 cells (Schafer *et al.*, 1996). Subcellular fractionation studies of .114 cells have demonstrated that DM is present at one-fifth of the levels of HLA-DR in the MIIC and at one-twenty third of the amount of HLA-DR in the cell as a whole (Schafer *et al.*, 1996). At endosomal pH, a sub-stoichiometric amount of DM (turnover number of 1 DM molecule per 3-12 DR molecules) efficiently mediated exchange of low affinity peptides, including CLIP, for antigenic peptides in an enzyme-like manner (Vogt *et al.*, 1996).

Studies with mutant mice (C57BL/6, 129/SV) which fail to express H2-M have been performed in order to elucidate the function of DM in MHC class II antigen presentation (Martin *et al.*, 1996; Miyazaki *et al.*, 1996; Tourne *et al.*, 1997).

Splenocytes from these animals were unable to present peptides derived from protein antigens to class II-restricted T cells and positive selection of $CD4^+$ T cells was reduced in these H2-M deficient mice (Martin *et al.*, 1996; Tourne *et al.*, 1997). In these animals, the levels of MHC class II expression ($I\text{-}A^b$) on the B-cell surface were normal but $I\text{-}A^b$ class II dimers were SDS-sensitive and they were found to be presenting CLIP predominantly (Martin *et al.*, 1996; Miyazaki *et al.*, 1996). Additionally, in DR3 transfected T2 cells, which are DM deficient, proteolysis of Ii was less efficient and DR3-CLIP complexes were generated much more slowly than in wild-type cells (Riberdy *et al.*, 1994). From these findings, it has been concluded that DM has several important functions, such as facilitating efficient proteolysis of Ii in the endocytic pathway, antigen presentation by MHC class II molecules and, ultimately, selective activation of $CD4^+$ T cells by MHC class II-mediated antigen presentation.

The requirement of HLA-DM or H2-M for effective MHC class II antigen presentation does not seem to be absolute however, since some MHC class II molecules, including DP4, DQ1, DR3 and $I\text{-}A^b$, require DM (or H2-M) for proper antigen presentation (Morris *et al.*, 1994; Green *et al.*, 1995; Sloan *et al.*, 1995; van Ham *et al.*, 1996; Miyazaki *et al.*, 1996) while $I\text{-}A^k$ does not (Stebbins *et al.*, 1996). However, for antigen presentation by $I\text{-}A^d$ class II molecules, the requirement for H2-M seems to be less stringent than in the case of presentation mediated by $I\text{-}A^b$. Stebbins *et al.* (1995) have shown that the formation of $I\text{-}A^d$ SDS-stable dimers and the dissociation of CLIP were not affected by the absence of DM in 9.5.3 cells, suggesting that $I\text{-}A^d$ class II antigen presentation may be DM independent. However, Weenink *et al.* (1996) have demonstrated that $I\text{-}A^d$ transfected T2 cells (T2.d), which were DM deficient, failed to form $I\text{-}A^d$ SDS-stable dimers and to present peptides derived from intact proteins to specific T cell hybridomas. Furthermore, $I\text{-}A^d$ molecules from these cells were predominantly

occupied with CLIP on the cell surface, indicating that DM (or H2-M) may be required for the I-A^d-mediated antigen presentation. After removal of CLIP by DM in the MIIC, the binding groove of the MHC class II molecules may be assumed to be temporarily empty. Stern *et al.* (1992) have reported that purified empty MHC class II molecules were not stable and tended to aggregate.

In summary, DM catalyzes dissociation of the CLIP from MHC class II molecules prior to peptide loading, a process which involves conformational changes in MHC class II molecules resulting from direct association of DM with the MHC class II molecules at lysosomal pH (Ullrich *et al.*, 1997). DM may stabilize empty MHC class II molecules in the MIIC by protecting them from aggregation until antigenic peptides are loaded (Denzin *et al.*, 1996). In addition to facilitating CLIP removal, DM also catalyzes the release of other self-peptides from the MHC class II molecules (Kropshofer *et al.*, 1996). This has led to the suggestion that DM has the potential to function as a "peptide editor" (van Ham *et al.*, 1996) by selecting high stability MHC class II/peptide complexes before these complexes are presented to T cells. van Ham *et al.* (1996) showed that DM catalyses the release of peptides which do not have appropriate anchor residues and, hence, no optimal binding motif from HLA-DR3. In this way, DM facilitates selection of peptides that bind with high affinity to the MHC class II molecules for eventual presentation to the immune system from the pool of available peptides (van Ham *et al.*, 1996).

1.5. Other molecules involved in MHC II antigen presentation

Since MHC class II α and β subunits are synthesized and form heterodimers in the ER, additional proteins which are resident in this compartment may interact with the components of MHC class II antigen presentation system. Calnexin is a resident protein of the ER which stabilizes MHC class I molecules until antigenic

peptides are loaded. Romagnoli *et al.* (1995) have reported that the ER-resident calnexin remains associated with Ii until the assembly with class II dimers to form a nonameric complex is complete. This result implies that calnexin may protect Ii from degradation in the ER until Ii assembles with MHC class II dimers.

Additionally, Liljedahl *et al.* (1996) have shown that HLA-DO (DO), which is encoded within the MHC class II region, is associated with DM throughout the endocytic pathway from immediately after synthesis in the ER to the MIIC. These authors demonstrated the existence of DO/DM complexes in lysosomal compartments, suggesting that DO may serve to modulate DM function in some way, which may have consequences for MHC class II antigen presentation.

1.6. Objectives of the Project

The general principles of MHC class II-mediated antigen presentation in human and mouse B cells are well understood, especially the roles of Ii in transport to endosomal compartments and as a molecular chaperone for MHC class II-mediated antigen presentation. Recently, a number of studies have been carried out on the role of DM in this context (Kropshofer *et al.*, 1997). However, the interactions of DM or mouse DM, H2-M, with other molecules during transport along the endocytic pathway and its functional mechanism of peptide exchange in MIIC remain unclear.

To investigate the role of H2-M in the mouse MHC class II pathway to over look in the human system, the experimental aims of this project were as follows:

A. To study the effect of different solution conditions on the co-immunoprecipitation of the molecules which are involved in I-A^d class II antigen presentation.

Protein complexes may have stabilities and solubilities which vary, depending on the detergents and pH used to lyse cells. The immunoprecipitation of proteins in the I-A^d class II pathway was investigated systematically with A20 cells in different solution conditions, such as varying the pH from 7 to 4 and the chaotropic strength of detergents (NP40 (strong) and digitonin (weak)) used for lysis of the cells. This investigation of a range of conditions for immunoprecipitation was critical for the extraction of both the maximum number and the maximum amount of proteins and their intact complexes in a given experiment.

B. To determine the intracellular association of H2-M with I-A^d class II molecules and Ii in a mouse B cell line.

Co-immunoprecipitation has been employed as the primary method to observe the association of proteins involved in MHC class II antigen presentation. The association of proteins with H2-M in A20 cells was determined by primary immunoprecipitation against the three molecules, I-A^d, Ii and H2-M, which have established roles in I-A^d class II antigen presentation (Weenink *et al.*, 1996). The intracellular association of H2-M with I-A^d or Ii was investigated by co-immunoprecipitation as a function of pH in order to detect differential stability of complexes of these molecules. Then, the pH-dependent complex stability of these molecules was compared to their co-immunoprecipitation in [^{35}S]-methionine pulse-chase experiments coupled with double immunoprecipitation and analysis by a combination of autoradiography and Western analysis. Additionally, the potential for physical association of H2-M with Ii in the absence of I-A^d was observed by analysis of co-immunoprecipitation from an I-A^d negative cell line (M12.C3).

Chapter 2

2. Materials and Methods

2.1. Cell lines

A murine, I-A^d expressing, B cell lymphoma cell line, A20, which has been derived from BALB/c mice (Weenink *et al.*, 1996) was used as a model antigen presenting cell throughout this investigation. Another BALB/c derived I-A^d negative cell line, M12.C3 (Glimcher *et al.*, 1985), was used in experiments to characterize the consequences of the absence of I-A^d upon the association of H2-M with Ii and any effect upon degradation of Ii.

2.2. Reagents

The source of specific reagents used for this experiments is detailed in this section, otherwise standard laboratory reagents were used. A20 and M12.C3 cell lines were maintained continuously in RPMI 1640 medium (Media Facility, JCSMR) supplemented with 10% inactivated foetal bovine serum (FBS, Trace), 0.05mM 2-mercaptoethanol (2-ME, BDH), 10mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Boehringer), 2mM L-glutamine (Sigma) and 100U/ml each of penicillin (Trace), neomycin (Sigma) and streptomycin (Sigma) at 37°C in the presence of 5% CO_2 enriched atmosphere. Prior to immunoprecipitations, methionine-free RPMI 1640 (Gibco BRL) was used in experiments employing [^{35}S]-methionine (Amersham) protein labelling in order to deplete intracellular methionine. Nonylphenoxy polyethoxy ethanol (NP40, Sigma), sodium dodecylsulfate (SDS, BDH), digitonin (Sigma), sodium chloride (BDH), citric acid (Sigma) and sodium phosphate dibasic (Mallinckrodt) were used for citrate-phosphate cell-lysis buffers, the compositions of which are listed in Table 2.1. For use as protease inhibitors, *N, N, N', N'*-ethylenediaminetetraacetate (EDTA) was purchased from Ajax and phenylmethylsulfonyl fluoride (PMSF),

Table 2.1. Composition of citrate-phosphate buffer different pHs *per* 100ml final volume.

pH	0.1M citric acid (ml)	0.2M sodium phosphate dibasic (ml)
4.0	30.7	19.3
5.0	24.3	25.7
6.0	17.9	32.1
7.0	6.5	43.6

aprotinin, antipain, leupeptin and pepstatin A were obtained from Sigma. For preclearing cell lysates and the subsequent immunoprecipitation of desired molecules, protein A sepharose CL-4B and protein A sepharose-6MB beads were purchased from Pharmacia. To elute precipitated molecules from protein A sepharose beads, SDS-PAGE sample buffers were used, which include SDS, *N*-tris[hydroxymethyl]aminomethane (Tris, Gibco BRL), glycerol (BDH), and either bromophenol blue or Coomassie blue G-250 (Pharmacia) and phenol red (Sigma) for use as tracking dyes. Where required, 5% 2-ME was used as a protein disulphide-reducing agent. Both [^{14}C]-rainbow protein markers and rainbow protein markers were purchased from Amersham to standardize protein sizes on a protein gel. Tris, *N*-tris[hydroxymethyl]methylglycine (Tricine, Sigma), SDS and glycine (Extran) were used as components of the running buffers in SDS-PAGE. In the majority of experiments, pre-cast 10-20% gradient Tris-Tricine gels (Novel Experimental Technology (Novex)) were employed. The X-cell II Mini-cell and companion protein blotting module (Novex) were used for SDS-PAGE and the transfer of proteins from the gels to membranes. Methanol for the transfer buffer was purchased from Ajax and nitrocellulose membranes were purchased from Pharmacia. *N*-hydroxysuccinimido biotin reagent (NHS-biotin) and fluorescein isothiocyanate (FITC) for biotinylation and fluoresceinylation of antibodies were obtained from Pierce. Additionally, non-fat milk powder, polyoxyethylenesorbitan monolaurate (Tween-20, Sigma), phosphate-buffered saline (PBS, Media Facility JCSMR), bovine serum albumin (BSA, Armour co.) and sodium azide were used in buffers for Western blotting and flow cytometric analysis.

2.3. Antibodies

The recognition site, the concentration used for the various experiments and the source of each primary antibody are listed in Table 2.2.1. For immunoprecipitation and re-immunoprecipitation K553, P4H5 and MKD.6, directed against H2-M, Ii and I-A^d, respectively, were used (Table 2.2.1). For Western analysis, biotinylated (bio)-M5/114 was used alone or in conjugation with bio-MKD.6 to identify I-A^d , In-1 was used for Ii identification and 1B9A or #104 were used for H2-M identification (Table 2.2.2). FITC-conjugated M5/114 was used to label cell surface I-A^d for flow cytometric analysis (Table 2.2.3). The concentration and source of secondary antibodies used for the Western analysis were listed in Table 2.3. Preimmune rabbit serum was used for preclearing cell lysates before immunoprecipitations.

2.4. The co-precipitation of associated molecules in the class II antigen presentation pathway

All immunoprecipitations and re-immunoprecipitations were repeated at least three times for a consistency, no significant differences were observed.

2.4.1. Metabolic labelling, immunoprecipitation and protein identification

A20 or M12.C3 cells (refer to Figure legends for cell numbers used in particular experiments) were washed and pre-incubated twice for 30 minutes at 37°C in methionine-free RPMI 1640 culture medium (Gibco BRL), containing the supplements described in Section 2.2. This was done to prime the cells for labelling by depriving them of intracellular methionine. After pre-incubation, cells were spun for 5 minutes at 1500rpm (revolutions *per* minute) in a centrifuge (C1000-S5, Jouan). Cells (pellets) were then labelled with [^{35}S]-methionine (10μCi/1 million cells) in fresh methionine-free RPMI 1640 medium (1ml/10

Table 2.2. Primary antibodies used for immunoprecipitation, re-immunoprecipitation, Western blotting and FACS assay.

Table 2.2.1. Antibodies used for immunoprecipitation and re-immunoprecipitation.

Antibody	Epitope/Comment	Concentration used	Source
MKD.6	anti-I-$A^d\beta$, $\alpha\beta$ dimer conformation dependent, purified, monoclonal	1/70 dilution of 5mg/ml	ATCC HB 3
P4H5	anti-Ii, recognises C-terminus of CLIP (residues 99-116) in Ii , purified, monoclonal	1/500 dilution of 250μg/ml	Reference: Mehringer *et al.*, 1991
K553	anti-H2-M, anti-serum against H2-M, unpurified, polyclonal	1/500 dilution as supplied	Dr. L. Karlsson

Table 2.2.2. Antibodies used for Western blotting.

Antibody	Recognition/Comment	Concentration used	Source
In-1	anti-Ii, recognises N-terminal residues 2-17 of Ii, purified, monoclonal	1/500-1/1000 of 0.1mg/ml	Reference: Mehringer *et al.*, 1991
1B9A	anti-H2-Mb, recognises H2-Mb, purified, monoclonal	1/500 dilution as supplied	Dr. L. Karlsson
#104	anti-H2-Ma, recognises H2-Ma, unpurified, polyclonal	1/1000 dilution as supplied	Dr. J. Trowsdale
bio-M5/114	anti-I-$A^d\beta$, recognises I-$A^d\alpha\beta$ and I-$A^d\beta$, purified, monoclonal	1/1000 dilution of ~1mg/ml	ATCC TIB 120

Table 2.2.3. Antibodies used for FACS assay.

Antibody	Recognition/Comment	Concentration used	Source
FITC-M5/114	same as bio-M5/114	1/2000 dilution of ~1mg/ml	ATCC TIB 120

Abbreviation: ATCC - American Type Culture Collection

Table 2.3. Secondary antibodies used for Western analysis.

Secondary antibody	**Primary antibody**	**Molecule to be recognised**	**Concentration**	**Source**
Goat anti- rat IgG-HRP	In-1 1B9A	Ii Mb	1/50,000 dilution of 0.8mg/ml	Pierce
Goat anti-rabbit IgG-HRP	#104	Ma	1/100,000 dilution of 0.8mg/ml	Pierce
Streptavidin-HRP	bio-M5/114 bio-MKD.6	I-$A^d\beta$, I-$A^d\alpha\beta$ I-$A^d\alpha\beta$	1/7,000 dilution of 5mg/ml	Pierce

Abbreviations: IgG, immunoglobulin G: HRP, horse radish peroxidase: bio, biotinylated

million cells) for 2 hours at 37°C in the presence of 5% CO_2 atmosphere. The labelled cells were washed sequentially four times in 20ml ice-cold PBS (137mM NaCl, 7mM $Na_2HPO_4.2H_20$, 2.5mM $NaH_2PO_4.H_2O$, pH7.4) by resuspending and centrifuging them again (as above) and then lysed in 200µl (*per*100 million cells) 1% NP40 lysis buffer (1% NP40, 0.1% SDS, 150mM NaCl, citrate-phosphate buffer) or 1% digitonin lysis buffer (1% digitonin, 150mM NaCl, citrate-phosphate buffer) pH7 to pH4 (see Table 2.1 for details), containing protease inhibitors (2mM EDTA, 1mM PMSF, 2µg/ml aprotinin and 10 µg/ml each of antipain, pepstatin A and leupeptin) for 15-20 minutes at 4°C by gentle rotation. Lysed cells were spun at 14,000rpm in a microcentrifuge (5415C, Eppendorf) for 20 minutes at 4°C to remove nuclei and other cell debris and the supernatant was collected. The lysate was precleared for 3 hours at 4°C by gentle rotation with total 8µl normal rabbit serum and 80µl protein A sepharose CL-4B or protein A sepharose-6MB beads. The beads were spun briefly, for 20 seconds at 2000rpm, in the microcentrifuge and the supernatant was collected. After preclearing, the lysate was subjected to immunoprecipitation by gentle rotation for 2 hours at 4°C with MKD.6, P4H5 or K553 in the presence of 30µl protein A sepharose-6MB beads for I-A^d, Ii or H2-M precipitation, respectively (see Table2.2.1 for antibody concentrations). After pelleting the beads (as above), the immunoprecipitates (beads) were washed 6 times in 1ml of the corresponding lysis buffer. In the case of digitonin lysis, the first 4 washes employed 0.05% digitonin but during the last 2 washes digitonin-free citrate-phosphate buffer was used to preclude digitonin smearing during protein electrophoresis. The precipitated molecules were eluted from beads sequentially, at first under non-denaturing conditions and, subsequently, by denaturing the samples. For non-denatured eluates, the beads were incubated for 25 minutes at 37°C in a Tris-Tricine gel loading buffer (see Section 2.6 for details). The beads were incubated for a second time for 5 minutes

at 100°C in the same loading buffer however which now contained 2.5% 2-ME for denatured samples. Non-denatured and denatured eluates were spun briefly to separate the protein supernatant from sepharose beads. Eluates were loaded on 10-20% Tris-Tricine gradient gels, alongside ^{14}C-labelled rainbow protein markers. Proteins were separated by electrophoresis and, subsequently, electroblotted onto nitrocellulose membranes. Details for gel electrophoresis and protein transfer onto membranes are described in Section 2.6. For radiographic analysis, membranes were exposed on an autoradiography film (X-OMAT, Kodak) and developed by an automated film processor (X-OMAT M20 processor, Kodak) and, in some experiments, they were exposed again on a phosphorimager screen for subsequent phosphorimager analysis (see Section 2.8 for details). For immunoanalysis the membranes were screened directly by immunoblotting, as described in Section 2.5. The primary antibodies used for Western analysis were bio-M5/114 with bio-MKD.6, In-1 and #104 for I-A^d, Ii and H2-M identification, respectively (see Table 2.2.2 for antibody concentrations). The secondary antibody conjugates anti-rat IgG-HRP (immunoglobulin G-horse radish peroxidase) or anti-rabbit IgG-HRP were used for the primary antibody In-1 or #104, respectively, while streptavidin -HRP was used with bio-M5/114 and bio-MKD.6 (see Table 2.3 for antibody concentrations). Western analysis using secondary antibody alone was done for the negative control of each primary antibody.

2.4.2. Pulse-chase experiment and re-immunoprecipitation

100 to 150 million cells were washed once and methionine depleted as described in Section 2.4.1. Cells were pulsed with 1 to 1.5mCi [^{35}S]-methionine in 10ml methionine-free RPMI 1640 for 30 minutes at 37°C in CO_2 rich atmosphere (5% CO_2) , then washed four times in 20ml ice-cold PBS to remove

extra [^{35}S]-methionine on the cells. Labelled intracellular methionine was chased for various periods, generally 0h, 3h, 6h and overnight, by incubating cells at 37°C in a 5% CO2 atmosphere in the culture medium described in Section 2.2 containing 10-fold excess of [^{32}S]-methionine. At the appropriate time point in the chase, the cells were washed twice and lysed, the cell lysate was precleared and then the desired molecules were precipitated. The sepharose beads were washed and eluted under non-denaturing conditions. Briefly, cells were washed twice with ice-cold PBS, lysed in 200µl 1% NP40 or 1% digitonin lysis buffer (only pH7 was used in these experiments) by gentle rotation for 20 minutes at 4°C and the supernatants were collected after centrifugation for 20 minutes at 14,000rpm at 4°C in the microcentrifuge. The lysate was precleared four times for 3 hours at 4°C with total 8µl normal rabbit serum in the presence of 80µl protein A sepharose CL-4B or protein A sepharose-6MB beads. After spinning the precleared lysate for 20 seconds at 2000rpm, supernatant was collected and I-A^{d}, Ii or H2-M was precipitated with MKD.6, P4H5 or K553, respectively (refer to Table 2.2 for antibody concentrations). Precipitates were washed six times in 1ml of the corresponding lysis buffer with the exception that 0.05% digitonin was employed for the first 4 washes and digitonin free citrate-phosphate buffer used for the last 2 washes of the digitonin lysed precipitate. In the pulse-chase experiments, the beads were extracted into sample buffer and eluates analyzed by SDS-PAGE. In the double precipitation experiments, immunoprecipitated molecules were washed six times and then eluted in 20µl of 1%SDS, 150mM NaCl, citrate-phosphate buffer pH7 for 2 hours at 37°C. After a brief centrifugation for 20 seconds at 2000rpm in the microcentrifuge, eluates were collected and the elution buffer was adjusted to 200µls of 1% NP40, 0.1% SDS, 150mM NaCl and citrate-phosphate pH7. Eluates were re-immunoprecipitated with K553 (anti-H2-M) from primary precipitates with P4H5 (anti-Ii) and P4H5 or

MKD.6 (anti-I-A^d) from primary precipitates with K553 in the presence of 30µl protein A sepharose-6MB overnight by rotating gently at 4°C. The sepharose beads, loaded with bound re-precipitates, were washed four times in 1ml of the corresponding re-precipitation buffer and, subsequently, re-precipitated molecules were analyzed in a reduced and denatured state only by eluting in Tris-Tricine SDS-PAGE gel loading buffer with 2.5% 2-ME and boiling for 5 minutes before analyzing by 10 - 20% Tris-Tricine gradient gels. Samples were electrophoresed and transferred onto nitrocellulose membranes as described in Section 2.6. Membranes were analysed by autoradiography followed by immunoblotting with bio-M5/114, In-1 and #104 for I-A^d, Ii and Ma identification as described in Section 2.5.

2.4.3. The labelling of M5/114 with biotin

I-A^d class II molecules precipitated from immunoprecipitations or re-immunoprecipitations were identified by using biotin conjugated M5/114 (bio-M5/114). The procedure for biotinylation of M5/114 was based on 'Current Protocol in Immunology (supplement 22, 5.3.6)' with modifications. For this, 500µl purified M5/114 (~1mg/ml) was dialyzed against biotinylation buffer (0.5M $NaHCO_3$, pH8.5) at 4°C with three changes for 2 days. A solution of NHS-biotin in dimethylsulfoxide (DMSO, 60µg/60µl) and that of the antibody were combined in a microcentrifuge tube which was wrapped with aluminum foil for protection from light. The mixture was incubated with rotation for 4 hours at room temperature and then, to remove unreacted NHS-biotin, it was dialyzed against 500ml PBS at 4°C with three changes for 2 days. The optimal concentration of bio-M5/114 required for identification of I-A^d class II molecules was determined by serial titration in Western blotting experiments with A20 cell lysates and was

determined to be a 1/1000 fold dilution of the dialysate from the biotinylation reaction (approx. 1mg/ml).

2.5. Direct identification of intracellular molecules by immunoblotting

In order to detect the Ma and Mb monomers (which constitute the heterodimer H2-M) in A20 cell lysates at different pH, cells were lysed either in 1% digitonin (for Ma identification) or 1% NP40 (for Mb identification) at both pH5 and pH7 and analysed both under non-denaturing and denaturing conditions either on 10-20% Tris-Tricine gradient gel (for Ma) or 12% Tris-glycine gel (for Mb) (see Fig.4.1 & 2 for conditions of each molecule detection). Cells (160,000) were washed once in ice-cold PBS as described in Section 2.4.1 and divided into four portions. Each portion of cells was lysed either in 40µl 1% NP40 or1% digitonin lysis buffer pH7 or pH5 containing protease inhibitors (see Section 2.4.1 for details) for 20 minutes by rotating gently at 4°C. Lysates were cleared from debris by spinning for 20 minutes at 14,000 rpm in a microcentrifuge (Eppendorf) at 4°C and the supernatants were collected. Then, 50% of each lysate was denatured by boiling for 5 minutes in Tris-Tricine gel loading buffer for analysis with Tris-Tricine or SDS-Laemmli buffer for analysis with Tris-glycine gels (see Section 2.6), in the presence of 2.5% 2-ME; the residual 50% was mixed with the same loading buffers without 2-ME or boiling. 10µl of each sample was loaded in a well of pre-cast 10 - 20% Tris-Tricine gradient gel (Novex) or 12% Tris-glycine gel and the loading was duplicated for the negative control of each molecule identification. Proteins were separated by electrophoresis and transferred onto nitrocellulose membranes by using the electro-blotting module (Novex). The methods for gel electrophoresis and transferring proteins onto the membranes are described in detail in Section 2.6. Membranes were pre-incubated in blocking

buffer (PBS, 5% low fat milk, 0.01% Tween 20) overnight at 4°C to block non-specific protein binding sites. Membranes were then incubated with primary antibody in the same buffer for 2 hours by gentle rotation at room temperature. The primary antibodies used for this immunoblotting are #104 and 1B9A for Ma and Mb detection, respectively, (see Table 2.2.2 for antibody concentration). To remove excess primary antibody, the membranes were washed three times, once for 15 minutes and twice for 10 minutes, in PBS containing 0.01% Tween 20 (PBS-T) at room temperature followed by incubation with secondary antibodies in PBS-T for 1 hour at room temperature. For secondary antibodies, anti-rat IgG-HRP was used for the primary antibody 1B9A and anti-rabbit IgG-HRP was used for the primary antibody #104. Membranes were washed again 3 times, using the same procedure as above and protein detection was carried out using the enhanced chemiluminescene (ECL) protein detection system (Amersham) following the manufacturers instructions. The chemiluminescent blots were exposed to a Kodak X-OMAT film and the film was developed by an automated film processor (Kodak).

To confirm the presence of Ii and H2-M in the I-A^d deleted cell line, M12.C3 cells, gel electrophoresis and Western blotting conditions were exactly the same as above with a few exceptions. The exceptions were that both M12.C3 and A20 cells were lysed in 1% NP40 lysis buffer at pH7 only and samples were analysed on the pre-cast 10-20% Tris-Tricine gradient gel. Also, In-1 and #104 antibodies were used for Ii and Ma detection respectively and secondary antibodies anti-rat IgG-HRP and anti-rabbit IgG-HRP were used for the primary antibodies In-1 and #104 (see Table 2.2.2 & 2.3 for concentrations of the primary and secondary antibodies). The method of Ii and Ma identifications in A20 and M12.C3 cell lysates was also described in detail in Fig. 6.2.

2.6. SDS-PAGE and protein transfer to a nitrocellulose membrane

Two different protein gel electrophoresis systems were used in the experiments in order to provide greater protein resolution, namely, 12% Tris-glycine SDS-PAGE and 10-20% Tris-Tricine SDS-PAGE. The Tris-glycine gels were used routinely and the 10-20% Tris-Tricine gradient gels (Novel Experimental Technology, San Diego, CA, USA) were used to detect peptides and low molecular weight proteins (2kDa peptides and above) because they provided better resolution of the low molecular weight species. For both systems, the proteins in the immunoprecipitates, re-precipitates or cell lysates were separated on the basis of eletrophoretic mobility of the protein-SDS complex which effectively depends on their sizes.

The procedure for the Tris-glycine SDS-PAGE was carried out using the Laemmli discontinuous buffer system (Laemmli, 1970). The gels were made freshly and constituted of 2 phases, an upper 5% polyacrylamide stacking phase and a lower 12% polyacrylamide resolving phase. Samples (immunoprecipitates or cell lysates) were either treated by boiling for 5 minutes with a reducing agent (2.5% 2-ME) in the Tris-glycine gel loading buffer (50mM Tris. HCl pH6.8, 2% SDS, 0.1% bromophenol blue, 10% glycine) for denatured samples or by incubation for 25 minutes at 37°C in the same loading buffer without 2-ME for non-denatured samples prior to loading on the gel. By denaturing samples, proteins are likely to be dissociated into their individual polypeptide subunits and aggregations tend to be minimized but non-denatured samples have the advantage that they are more likely to preserve protein complexes intact on the gel. Gels were run vertically in Tris-glycine gel electrophoresis buffer (25mM Tris, 250mM glycine pH8.3, 0.1% SDS), at 100V until the bromophenol blue dye reached the end of the stacking gel and then at 150V for electrophoresis through the resolving gel.

The procedure of electrophoresis in 10-20% Tris-Tricine gradient gel was carried out using the pre-cast Novex protein gel electrophoresis system. Gels consisted of only one polyacrylamide phase but the concentration of the polyacrylamide is 10% at the top of the gel and gradually higher to 20% at the bottom. The samples were applied to the gels in reducing or non-reducing conditions in a sample loading buffer (450mM Tris. HCl pH8.45, 4% SDS, 12% glycerol, 0.0075% Coomassie blue and 0.0025% phenol red) and gels were run with the same buffer in both tanks (0.1M Tris, 0.1M Tricine and 0.1% SDS) at a constant 125 volts. The electrophoretically separated proteins were electro-transferred onto nitrocellulose membranes for immunoblotting (Western blotting). For the transfer of proteins, an electro-blotting module (NOVEX) was used. On the bottom cathodic plate, 3 porous pads and 1 piece of Whatman 3MM paper that have been soaked in a transfer buffer (39mM glycine, 48mM Tris, 20% methanol) were placed. The protein gel was placed on the filter paper and a nitrocellulose membrane, which had previously been soaked in transfer buffer, was laid on the gel, taking care to avoid air bubbles. Finally, a pre-soaked piece of filter paper, 3 to 4 pads and the anodic plate were applied sequentially above the membrane. The assembled module containing the gel and membrane was lodged in an electrophoresis tank (Novex) and transfer of proteins from the gel to the membrane was carried out in the transfer buffer for 1 hour 30 minutes at 30 Volts. After electrophoresis, the membrane was removed from the sandwich and it was used for immunoblotting directly (see Section 2.5) or dried and exposed on a film if proteins on the membranes had been radio-labelled.

2.7. Fluorescence-activated cell sorter (FACS) analysis

2.7.1. Flow cytometric analysis of I-A^d cell surface molecules

The purpose of flow cytometry in this experiment was for analyzing the expression of cell surface molecules. The fluorescence intensity produced by fluorescence-labelled antibodies that bind to specific cell surface molecules is measured. A cell is monitored individually as it flows through a detection region illuminated by a laser beam.

A20 and M12.C3 cells in a 96 well round bottom microtiter plate (20,000 cells/well, Nunc) were washed once in 200μl of cold FACS medium (1% BSA and 0.05% sodium azide in PBS) by resuspending and centrifuging them for 5 minutes at 1500rpm in a centrifuge (C1000-S5, Jouan) at 4°C. The supernatant was removed and washed cells were stained with 0.5μg/ml fluorophore-conjugated antibody (FITC-M5/114, see below) in the FACS medium by incubating them for 45 minutes on ice. The cell suspension was centrifuged again as above and the supernatant was removed. Then stained cells were washed three times in 200μl FACS medium to remove excess of the antibody and dead cells were stained by 1μg/ml propidium iodide (PI) in the FACS medium for 5 minutes on ice. Cells were again washed twice in 200μl FACS medium after PI staining and then resuspended in 400μl FACS medium to be analyzed by a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA). The same process as above was repeated except without staining cells with the antibody for a negative control for the antibody staining. For each sample, the fluorescence intensity of 10^4 viable cells were scanned and dead cells were excluded from the counts.

2.7.2. Conjugation of M5/114 with fluorescein isothiocyanate (FITC)

Conjugation of M5/114 with FITC was done according to the procedure described in 'Current Protocol in Immunology (supplement 16, 5.3.5)'. Briefly,

purified 500µl M5/114 (approx.1mg/ml) in PBS containing 0.05% sodium azide was dialyzed against 500ml FITC labeling buffer (0.05M sodium borate, 0.2M NaCl pH9.2) at 4°C with three changes over 2 days. The dialysed antibody was conjugated with 10µl of FITC in DMSO (5mg/ml) in a microcentrifuge tube for 2 hours at room temperature with continuous inversion. The tube containing antibody and FITC was wrapped with a piece of foil to block any light. Unbound FITC was then removed by dialyzing the conjugated antibody against 500ml PBS at 4°C with three changes for 2 days. The optimal concentration of FITC conjugated M5/114 required to stain surface I-A^d was determined by serial titration in a FACS assay. The optimal concentration range obtained was at 1/200 to 1/2000 dilution of the dialysed antibody conjugate (approx. 0.5 to 5µg/ml).

2.8. Phosphorimager analysis

Phosphor-storage technology provides another system for analysis of radio-labelled molecules and it is useful for a quantitation of the labelled molecules. After exposing radio-labelled proteins on a nitrocellulose membrane to an autoradiography film (see Section 2.4.1), the membrane was exposed again to a phosphorimager screen for one day for relative quantitation of labelled proteins. The screen was scanned using a PhosphorImager (Molecular Dynamics). Autoradiographs of [^{35}S]-labelled protein electroblotts were analysed with ImageQuant Software version 3.0.

Chapter 3

3. Characterization of the immunoprecipitation of MHC class II-associated molecules

3.1. Introduction

Traditionally, immunoprecipitation of molecules involved in the MHC class II antigen presentation pathway, such as I-A^b, I-A^k and Ii, has been done in a strong non-ionic detergent, such as NP40, at pH7 to 8 (Bikoff *et al.*, 1995; Busch *et al.*, 1996; Stumptner *et al.*, 1997). The association of I-A^k, I-A^d, I-E^k and I-E^d with Ii is strong enough to be stable in these detergent solutions resulting in co-immunoprecipitation (Germain and Hendrix, 1991). However, Sanderson *et al.* (1996) have reported that complexes of HLA-DR and HLA-DM were detected after lysis in digitonin (a non-ionic, weakly chaotropic detergent) at pH5 only, neither in NP40 lysis at pH5 nor at pH7 in either detergent. This observation suggests that the co-precipitation of proteins in the MHC class II pathway should be systematically investigated.

Detergents of different chaotropic strengths lyse cells with different abilities. Using a weak detergent for cell lysis and subsequent immunoprecipitation has the advantage that it may result in co-precipitation of weakly associated molecules that would be dissociated and, thus, not detected in a strong detergent. However, the use of a strong detergent in cell lysis and subsequent immunoprecipitations also may have some advantages over the weak detergent, namely, the disruption of lipid membrane and the solubilization of antigens may be more efficient, along with washing precipitated immune complexes with greater stringency.

Protein complexes may have different stability at different pH. During the trafficking associated with maturation, MHC class II molecules experience a range of acidity from a neutral pH within the ER and then a gradual decline of pH along the endocytic pathway; which is pH6 in the Golgi complex, pH5 to 6 in endosomes

and between pH4.6 to 5.0 in the lysosomes of intact cells (Mellman *et al.*, 1986). By using solution conditions which represent only a sub-set of the possible intracellular pH conditions to lyse cells for immunoprecipitation, pH-sensitive protein complexes from specific compartments may not be detected. Protein complexes which exist in cell environments at pH7 may be dissociated at pH5 or, conversely, protein complexes which are stable at pH5 may not be stable at pH7, so their association may not be detected at every pH. In addition to the different stability of protein complexes at different environmental acidity, protein solubility may also be dependent upon pH. For example, proteins (or complexes) soluble in one specific pH environment may be less soluble or not soluble at another pH. As a consequence, comparison of cell lysis and immunoprecipitation at differing acidity may reveal reduced abundance or even complete loss of immunoprecipitated material. Therefore, because different protein complexes have different stabilities and different proteins have different solubilities depending on detergents and pHs, the use of a range of detergents and pH to lyse cells may be critical to extract both the maximum number and a maximum amount of proteins and their intact complexes in a given immunoprecipitation experiment.

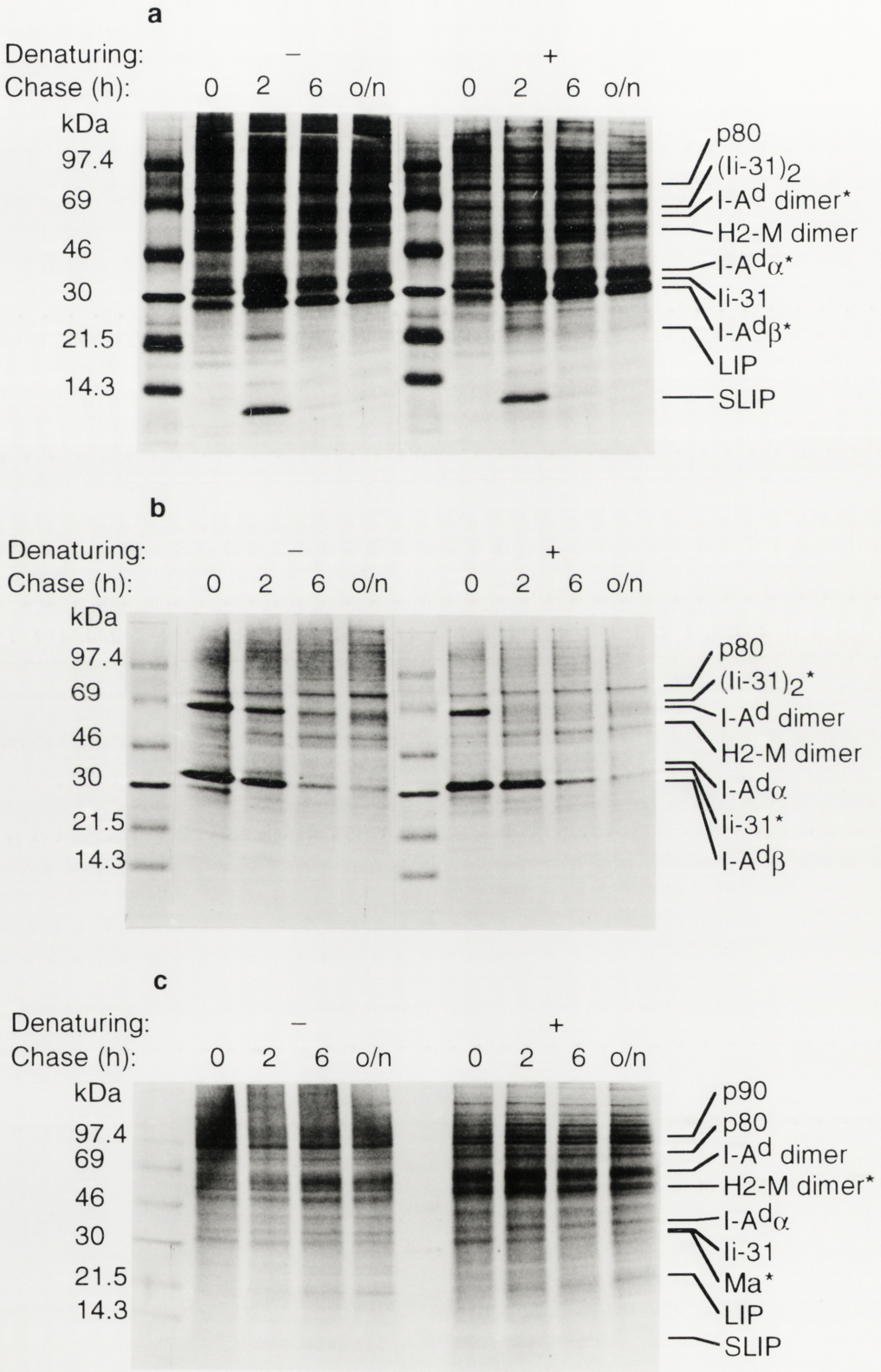
By lysing cells and performing immunoprecipitation in different strengths of detergent at varying pH, the sensitivity of various molecular interactions to these factors may be revealed. Additionally, these properties may reflect the origin and functional role of protein complexes in different intracellular compartments. In pursuit of this, development of an experimental immunoprecipitation regime was undertaken. Cell lysis and immunoprecipitation were performed at four different acidities, *viz.* pH7, 6, 5 and 4. To observe any effect of detergent on the immunoprecipitation of MHC class II-associated molecules, NP40 was used to lyse cells with a stronger chaotropic detergent and digitonin was used to lyse cells with a weaker chaotropic detergent. Since, to date, the majority of

immunoprecipitations have been done at a neutral pH in NP40 lysis, this development of experimental methods in different solution conditions provides an extended investigative tool for the precipitation of MHC class II-associated molecules. Furthermore, it also provides information about the stability of molecular complexes involved in MHC class II antigen presentation and their functional association in different compartments of the cell.

3.2. Identification of class II-associated molecules

Prior to the observation of any pH and detergent effects on the co-precipitation of MHC class II-associated molecules, the identification of molecules which are involved in I-A^d class II antigen presentation in A20 cells was established. The glycoproteins Ii, H2-M, and I-A^d class II are the major molecules involved in I-A^d class II antigen presentation. The identification of these molecules has been achieved by immunoprecipitation of each molecule with a specific antibody at different time chases (0h, 2h, 6h and overnight) after labelling with [^{35}S]-methionine. Cells were lysed in 1% NP40 lysis buffer at pH7 (see Section 2.2 for the composition of the buffer), then I-A^d, Ii and H2-M and associated molecules were precipitated, re-solubilised with both non-denaturing and denaturing conditions and analysed using 10-20% gradient gels for Tris-Tricine SDS-PAGE.

The method and conditions for these immunoprecipitations have been described in Section 2.5 except that a different number of cells and only NP40 detergent were used in these immunoprecipitations. Briefly, A20 cells (see Fig. 3.1 legend for the cell number) were washed in ice-cold PBS and pre-incubated twice for 30 minutes in methionine-free RPM1640 at 37°C in 5% CO_2 atmosphere to deplete of intracellular methionine. Cells were pulsed with [^{35}S]-methionine for 30 minutes at 37°C, then washed four times with cold PBS. The cells were divided into four portions and directly after [^{35}S]-labelling one portion of cells was


a
Denaturing: – +
Chase (h): 0 2 6 o/n 0 2 6 o/n
kDa
97.4
69
46
30
21.5
14.3
p80
(Ii-31)2
I-Ad dimer*
H2-M dimer
I-Adα*
Ii-31
I-Adβ*
LIP
SLIP
b
Denaturing: – +
Chase (h): 0 2 6 o/n 0 2 6 o/n
kDa
97.4
69
46
30
21.5
14.3
p80
(Ii-31)2*
I-Ad dimer
H2-M dimer
I-Adα
Ii-31*
I-Adβ
c
Denaturing: – +
Chase (h): 0 2 6 o/n 0 2 6 o/n
kDa
97.4
69
46
30
21.5
14.3
p90
p80
I-Ad dimer
H2-M dimer*
I-Adα
Ii-31
Ma*
LIP
SLIP

subjected to lysis for 20 minutes in NP40 lysis buffer at pH7 (see Section 2.2 for the composition of the buffer) at 4°C. The [^{35}S]-labelled methionine pulse in the other three portions of cells was chased for 2h, 6h or overnight by incubating in ten fold excess of [^{32}S]-methionine-rich RPMI medium (see Section 2.2). At each time point, the chased cells were washed twice in cold-PBS and lysed as described above. The cell lysates were precleared with normal rabbit serum in the presence of protein A sepharose-6MB beads for 3h at 4°C. The precleared lysates and beads were centrifuged for 20 seconds at 2000rpm in the microcentrifuge and the supernatants were collected. The precleared cell lysates from each of the four time points in the chase were divided into three portions and each portion of the lysates was immunoprecipitated with MKD.6 (anti-I-A^{d}), P4H5 (anti-Ii) or K553 (anti-H2-M) (see Table 2.2.1 for the antibody concentration) with protein A sepharose-6MB for 2h at 4°C. The bead suspensions were pelleted as above and the beads were collected. The beads were now washed six times in the lysis buffer. Immunoprecipitates were eluted under non-denaturing conditions with Tris-Tricine gel loading buffer (see Section 2.6) by incubation for 25 minutes at 37°C. Beads were spun and the eluates were transferred into other tubes. The beads were extracted a second time, under reducing and denaturing conditions, in the same SDS-PAGE loading buffer as above but now containing 2.5% 2-ME, with boiling for 5 minutes. Non-denatured and denatured sample eluates were loaded separately on 10-20% Tris-Tricine gradient gels and electrophoresed before analyis by autoradiography.

Each immunoprecipitated or co-precipitated molecule was identified either specifically by comparison with reports of proteins immunoprecipitated previously under similar conditions or assigned a code name; proteins (p) which were present both under non-denaturing conditions and not disturbed by denaturing conditions

or complexes (c) which were present under non-denaturing conditions but disturbed by denaturing conditions, followed by the size (kDa) of the band.

As shown in Fig. 3.1a, 33kDa and 28kDa bands were precipitated with MKD.6 at all chase times in A20 cells. The antibody MKD.6 is a monoclonal anti-I-$A^d\beta$ antibody which immunoprecipitates I-A^d dimers and associated molecules. These 33kDa and 28kDa bands had been assigned as I-A^d class II α and β chains, respectively, in the immunoprecipitation of the same cell line (A20) with the same antibody (MKD.6) by Weenink *et al* (1996). In their immunoprecipitation, the I-A^d α and β chains were seen clearly at all chase times also. In both studies, the intensity of both bands was dominant compared with other bands which is to be expected since these immunoprecipitations have been done with the anti-class II antibody.

After non-denaturing extraction, a molecule giving a band between 60kDa and 55kDa was precipitated weakly at 0h chase but was precipitated in greater abundance as the chase time was longer and, indeed, the most at the overnight chase (Fig. 3.1a). The band was not fully dissociated under denaturing conditions. These observations are in concordance with the band being derived from I-A^d $\alpha\beta$ dimers as discussed by Weenink *et al.* (1996).

A strong 31kDa protein band was precipitated by MKD.6 clearly at 0h chase, weakened at 2h chase and disappeared after the 6h chase period. According to the interpretation of Weenink *et al.* (1996), this 31kDa size molecule is Ii which demonstrates a strong association with I-A^d class II molecules by virtue of its co-precipitation with anti-I-A^d immunoprecipitation in the strong detergent NP40. The observation that the intensity of 31kDa Ii (Ii-31) band was weakened after the longer chase times is consistent with the current understanding that Ii is degraded by proteases in endosomal compartments (Blum and Cresswell, 1988; Maric *et al.*, 1994).

A 60kDa band was precipitated by MKD.6 immediately after the [^{35}S]-methionine pulse and is visible in the non-denatured eluates, but was largely dissociated in the denaturing extraction. A molecule of similar size has been co-purified with HLA-DR from Swei cells and it has been interpreted as a disulfide-linked dimeric form of Ii by Roche *et al.* (1991).

Finally, 22kDa and 10kDa molecules were precipitated after the 2h chase and these were detectable in both non-denatured and denatured extracts. Schafer *et al.* (1996) have found molecules of a similar size which co-purify with HLA-DR3 from leupeptin treated .114 cells and they identified the 22kDa band as being leupeptin-induced Ii peptides (LIP) and the 12kDa band as being small leupeptin-induced Ii peptides (SLIP) by immunoblotting with the anti-Ii antibody, PIN.1. Also, Nguyen *et al.* (1989) have precipitated similar size molecules (21kDa and 10kDa) by immunoprecipitation of leupeptin treated Raji cells with the anti-class II antibody VIC-Y1 and they assigned the 21kDa band as LIP and the 10kDa band as SLIP. If both 22kDa and 10kDa bands in Fig. 3.1a are Ii fragments then their appearance at 2h chase is expected to parallel Ii (31kDa) degradation by leupeptin-sensitive proteases in the acidic environment of endosomal compartments. The fact that 31kDa Ii intensity was much reduced at 2h chase, while 22kDa and 12kDa size Ii fragments have appeared at the same time is good evidence of both Ii degradation in endosomal compartments and the identification of these bands as being LIP and SLIP.

Other notable bands in the anti-I-A^{d} immunoprecipitations (Fig. 3.1a) were 50kDa and 80kDa molecules which were not dissociated under the denaturing conditions. The 50kDa molecule may correspond to H2-M dimers because this band is the same size as H2-M dimers identified by immunoblotting from Ii precipitations in digitonin lysis at pH7 and pH6 (see Fig 4.5 and Section 4.3 for details). A molecule of 80kDa size was precipitated with MKD.6 at all chase times

and under all bead elution conditions, this molecule remains unidentified. It was equally abundant in non-denatured and denatured samples, so it was called p80 (protein 80kDa size).

In immunoprecipitations of Ii by the anti-Ii monoclonal antibody (mAb) P4H5 (Fig. 3.1b), a 31kDa band was precipitated most abundantly. This band was also present in the anti-I-A^d immunoprecipitations (see Fig. 3.1a). It was not dissociated under denaturing conditions and has been identified as Ii-31. A band of this size was consistent with the results of Germain and Handrix. (1991), who immunoprecipitated Ii-31 with the same antibody (P4H5) from spleen cells of CBA mouse. This Ii-31 band was the strongest at 0h chase and became weakened progressively at longer chase times. This progressive weakening of the Ii band intensity may be rationalised as being caused by Ii degradation in the endosomal compartments as explained above. In the immunoprecipitations by Germain and Handrix (1991), MHC class II α and β chains were identified above and below Ii-31, respectively, and apparently identical bands were seen also in this immunoprecipitation (Fig. 3.1b). The result that I-A^d class II α and β chains were co-precipitated from Ii immunoprecipitation in NP40 implies a strong association of MHC class II molecules with Ii.

Another dominant band in Fig. 3.1b was seen at 60kDa size which was somewhat dissociated under denaturing conditions. The intensity of this band was strong at 0h chase and much weakened as the chase time was longer in a manner parallel to Ii-31. This band was assigned as being a dimer of Ii because a band of same size was identified subsequently to be Ii-immunoreactive by Western analysis with the anti-Ii antibody In-1 directly from cell lysates under non-denaturing conditions (see Fig. 6.2a). In precedence of this, Germain and Handrix (1991) observed a band of this size from anti-Ii immunoprecipitations and, further, it was not detected from immunoprecipitations from which Ii had been precleared.

Furthermore, a molecule of this size was co-purified with HLA-DR5 from B-LCL Swei cells by Roche *et al.*(1991) and was interpreted as a dimer of Ii disulphide linked at the cytoplasmic terminus.

Proteins which gave bands at 33kDa and 28kDa were precipitated with P4H5 at 0h chase which correspond to the sizes of I-$A^d\alpha$ and I-$A^d\beta$, respectively, in the anti-I-A^d immunoprecipitation (see Fig. 3.1a). These molecules were visible under both non-denaturing and denaturing conditions and the intensity of [^{35}S] in both molecules was reduced at longer chase times, especially at 6h and overnight chases. By contrast, bands at the same size of I-A^d heterodimers (both compact and floppy forms) between 60kDa and 55kDa were precipitated in slightly greater abundance after the longer chase periods when analyzed under non-denaturing conditions and the intensity of these bands was reduced but still visible under denaturing extraction conditions. The strong association between Ii and I-A^d was seen by co-precipitation of I-A^d monomers and dimers with Ii in NP40 lysis.

A molecule of 50kDa, the size of the H2-M dimer, was co-precipitated by P4H5 at 0h chase under both non-denaturing and denaturing conditions and the intensity of this molecule increased after 2h and remained sensibly constant thereafter.

Anti-H2-M immunoprecipitations were performed using the antibody K553 and the results were shown in Fig. 3.1c. Since this antibody is an unpurified anti-serum and H2-M is present in low amounts in cells, these immunoprecipitation results showed higher background and a weaker signal for bands at the size of H2-M monomer, Ma, compared with I-A^d α and β or Ii in immunoprecipitations of I-A^d or Ii. A band at 31kDa was visible very close to Ii-31 at all chase times independent of denaturing conditions. This band at 31kDa has been confirmed as being Ma by Western analysis with the anti-Ma antibody #104 from H2-M immunoprecipitates in digitonin lysis at pH7 (see Fig. 4.3). Also, a 31kDa band

was seen in the identification of Ma in B10.M splenocyte lysates by Karlsson *et al.* (1994) precipitated with the same antibody. However, a band at 27kDa corresponding to Mb which had been shown in the work of Karlsson *et al.* (1994) was not seen clearly in these immunoprecipitations. The absence of Mb in these immunoprecipitations might be caused by differences between the cell lines or simply different cell numbers. Bands at 50kDa and between 60kDa and 55kDa are most likely to be H2-M dimers and I-A^d dimers, respectively (see above for details). Bands of 33kDa were seen consistently at all chase times in both non-denatured and denatured samples and were assigned as I-A^d α monomers.

The faint bands at 22kDa and 10kDa were co-precipitated with H2-M at later time-points in the chase (after 2h) but the intensity of these bands was not strong (Fig. 3.1c). These were probably the small amount of Ii intermediates associated with H2-M, either directly or indirectly through the medially of I-A^d. A high molecular weight band, denoted p80, was precipitated with H2-M also, as was observed for the other immunoprecipitations (*i.e.* anti-I-A^d and anti-Ii immunoprecipitations), weakly under non-denaturing and more strongly under denaturing conditions. A strong band at 90kDa was co-precipitated consistently with H2-M at all chase times, independent of the conditions of bead extraction and many molecules at higher size than 90kDa were precipitated also. These were especially visible under denaturing extraction conditions but it is not clear whether they were H2-M associated molecules involved in the MHC class II antigen presentation or non-specific protein complexes precipitated by the unpurified antibody, K553.

Overall, many of the molecules immunoprecipitated or co-precipitated with I-A^d, Ii and H2-M are consistent with the current understanding of the associations of molecules in the MHC class II pathway which seem to be strong enough to be effectively co-precipitated in the strong detergent NP40. However, other

complexes may exist *in vivo*, as demonstrated by Sanderson *et al.* (1996) in Raji cells who have shown that the DM-DR association was detected in digitonin at pH5 only. The effect of detergent and pH are discussed in the subsequent sections of this chapter.

3.3. The effect of detergent on immunoprecipitation of MHC class II-associated molecules

Immunoprecipitations designed to investigate detergent effect upon co-precipitation of MHC class II -associated molecules were performed as described in Section 3.2, with the exceptions that fewer cells were used and two detergents, either digitonin or NP40, were used to lyse the cells in parallel experiments. The cells were pulsed with [^{35}S]-methionine and the labelled proteins were chased for various periods. Then the cells were divided into 2 pools for each time-point, which were lysed in either NP40 or digitonin, at pH7. Antibodies against I-A^{d}, Ii or H2-M were used individually in three different immunoprecipitations and precipitated molecules were extracted from the beads sequentially under non-denaturing (non-reducing and unboiled) and denaturing (reducing and boiled) conditions.

Briefly, A20 cells were washed and intracellular methionine was depleted by incubation twice for 30 minutes in methionine-free RPMI 1640. Cells were then pulsed with [^{35}S]-methionine for 30 minutes at 37°C and washed with ice-cold PBS four times. Pulsed cells were divided into four portions and chased over increasing time increments (0h, 2h, 6h or overnight) with 10 fold excess of [^{32}S]-methionine in the culture medium (see Section 2.4.1 for details of culture medium). Chased cells were washed and divided again into two portions for lysis in 1% NP40 lysis buffer or 1% digitonin lysis buffer at pH7 only. The cells were lysed as described in Section 3.2 and the precleared lysates were then divided again into three

portions for precipitation with different antibodies. Each portion of the lysates were precipitated with MKD.6, P4H5 or K553 in the presence of 30μl protein A sepharose-6MB beads for 2 hours at 4°C. After precipitation, the beads were washed and the precipitates were eluted in 20μl Tris-Tricine gel loading buffer for 25 minutes at 37°C (see Section 2.4.2 for details). These eluates were transferred into other tubes and comprised the samples obtained under non-denaturing conditions. The beads were extracted for a second time by boiling in 20μl of the same Tris-Tricine gel loading buffer containing 2.5% 2-ME for the denatured samples. Proteins in the non-denatured and denatured samples were separated by 10-20% Tris-Tricine gradient gel eletrophoresis and transferred onto nitrocellulose membranes before analyzing by autoradiography.

There were some detergent-related differences in the molecules precipitated from the two different lysates. The results of the immunoprecipitations with anti-I-A^d antibody MKD.6 in both detergents at each time point are shown in Fig. 3.2 and the molecules which were precipitated differentially in the two detergents at different time chases were summarized in Table 3.1. In the non-denatured samples (Fig. 3.2a), a band at 120kDa (c120) was seen more in digitonin lysis than in NP40 lysis at 0h chase. Also, the I-A^d dimer at 60kDa (Weenink *et al.*, 1996) was precipitated more abundantly following digitonin lysis than in NP40 lysis throughout the chase period. Under denaturing conditions (Fig.3.2b), the band at 120kDa was dissociated and a band named as p43, most distinct at 0h chase time, was more abundant in digitonin lysis than in NP40 lysis. By contrast, lower molecular weight proteins visible after denaturation, such as p27 and p18, were seen more abundantly in NP40 lysis than in digitonin lysis throughout the chase (Fig 3.2b).

In immunoprecipitations with the anti-Ii antibody P4H5, no striking differences in the molecules precipitated with lysis in different detergent were detected under

Table 3.1. Summary of molecules which precipitated differentially in different detergents (according to Fig. 3.2-4).

Antidody	MKD.6				P4H5				K553			
Figure	3.2				3.3				3.4			
Denaturing	-		+		-		+		-		+	
Detergent	NP	dig	NP	dig	NP	dig	NP	dig	NP	dig	NP	dig
Molecule size (kDa) / chase time (h)		120/0 60/all	27/all 18/all	43/0			28/all 27/all 25/all 18/all		220/all 76/all	250/0, o/n 100/0 43/0, o/n 31/0 22/0	76/all	220/0, o/n 100/0 43/0, o/n 31/0 22/0

Abbreviations: antibody - antibody used for immunoprecipitations
detergent - detergent used for cell lysis
molecule - molecule precipitated
NP - NP40
dig - digitonin
all - at all times
o/n - overnight

non-denaturing conditions (Fig 3.3a). However, in the denatured samples (Fig 3.3b) molecules smaller than 30kDa were seen to be more abundant in NP40 lysis than in digitonin lysis at all chase times once again (*cf.* Fig. 3.2b).

Immunoprecipitations with the anti-H2-M antibody K553 (Fig 3.4) showed the greatest difference in the molecules precipitated as a function of the two different detergents employed. Greater numbers of bands or a higher concentration of the same band were found in the digitonin lysates compared with the corresponding NP40 lysates. This may indicate that intracellular associations of H2-M are much weaker than the associations of I-A^d or Ii. In the non-denatured samples from H2-M precipitations (Fig 3.4a), high molecular weight bands at 250kDa were seen in digitonin only at 0h chase and more abundantly in digitonin than in NP40 at the overnight chase. A band at 220kDa was seen only in NP40 lysis throughout the chase and, conversely, p100 at 0h chase was seen in higher concentration in digitonin than in NP40 lysis. The band at 76kDa was stronger in NP40 lysis than in digitonin lysis at all chase times both with and without denaturation, reflecting the differences in the ability of the detergents to lyse cells and solublise proteins and their complexes. Furthermore, an intense band at 43kDa in digitonin lysis at the 0h time point and overnight chase in the non-denatured samples showed how important the choice of detergents for immunoprecipitations may be. Additionally, p31 was seen as a stronger band at 0h chase in digitonin than in NP40 lysis and, also, p22 was seen in digitonin lysis at 0h chase in both the unboiled and boiled samples and had disappeared at 2h and 6h, then re-appeared at overnight chase. The pattern of p22 appearance at different time chases is consistent with the pattern of p43 appearance suggesting p22 may be a monomer of p43. In the denatured extracts from anti-H2-M immunoprecipitations (Fig 3.4b), the 250 and 220kDa bands in the non-denatured samples were partially dissociated. Instead, an H2-M dimer size band, p50, was much stronger, especially at 0h chase in both

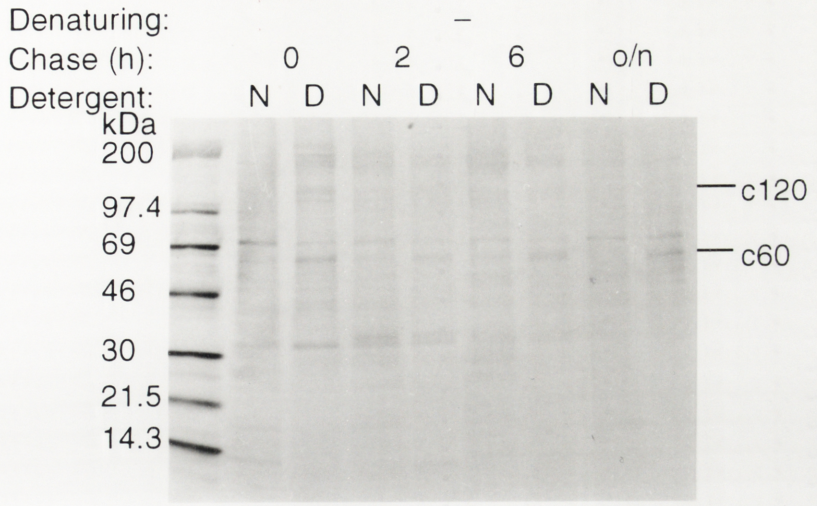
Fig. 3.2-4. Effect of different strengths of detergents on immunoprecipitation.

Molecules were precipitated differentially in NP40 and digitonin lysis in the pulse-chase experiments with MKD.6 (**3.2**), P4H5 (**3.3**) or K553 (**3.4**).

A20 cells (0.6 million cells/well for I-A^d or Ii, 2.5 million cells/well for H2-M) were washed and depleted of intracellular methionine, then the cells were pulsed with [^{35}S]-methionine and labelled molecules were chased for different times (0h, 2h, 6h or overnight) as in Fig. 3.1. Chased cells were washed two times in 20ml chilled PBS, divided again into two portions to be lysed in either 1% NP40 or 1% digitonin lysis buffer at pH7. Cells were lysed and precleared, then I-A^d, Ii or H2-M and associated molecules were precipitated with MKD.6, P4H5 or K553, respectively, as described in Fig. 3.1. Sepharose beads (and bound precipitates) were washed in 1ml of corresponding lysis buffer. However, 0.05% digitonin was used for the first four washes and no digitonin was used for the last two washes in the digitonin lysis washing buffer. The washed precipitates were eluted sequentially under non-denaturing (**3.2a**, **3.3a** & **3.4a**) and then denaturing conditions (**3.2b**, **3.3b** & **3.4b**) and the proteins in the precipitates were separated by 10-20% Tris-Tricine gel electrophoresis. The proteins separated on the gel were transferred onto a nitrocellulose membrane to be analysed by autoradiography. The method of protein extraction, gel electrophoresis and protein transfer is the same as in Fig. 3.1.

Abbreviations: -, non-denatured: +, denatured: o/n, overnight: N, NP40: D, digitonin: p, polypeptide: c, complex.

Fig.3.2a



b

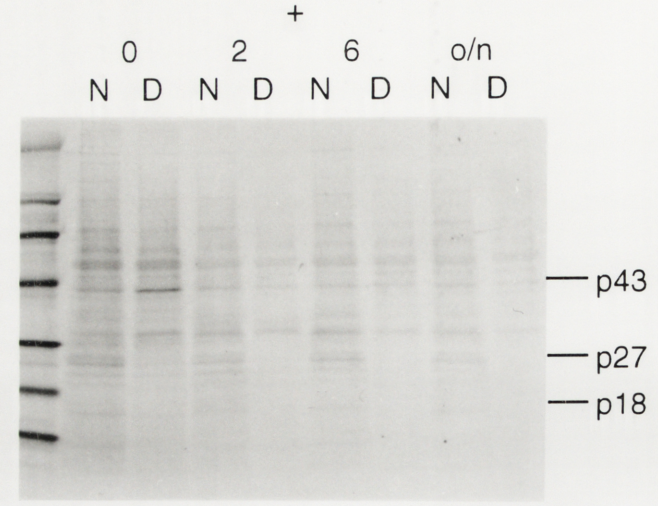
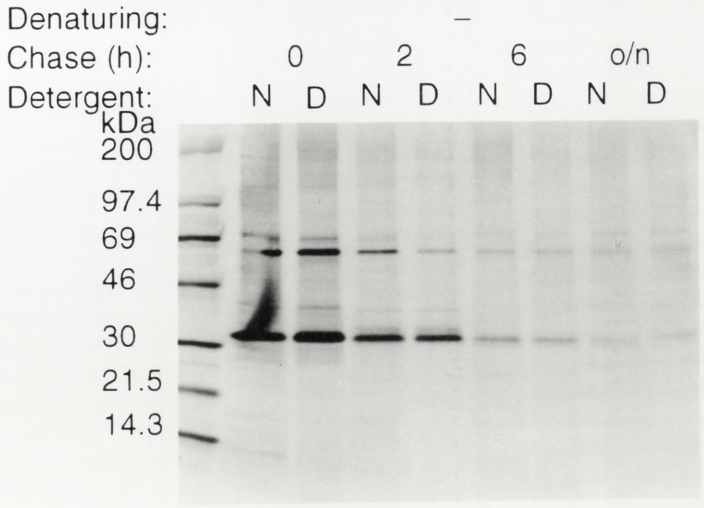


Fig.3.3a



b

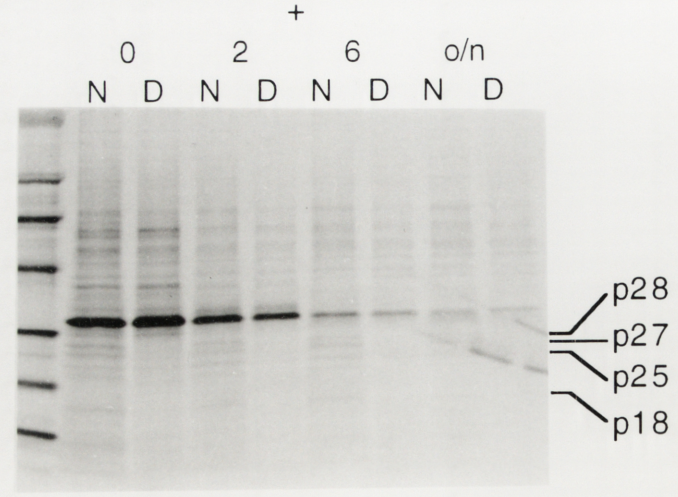
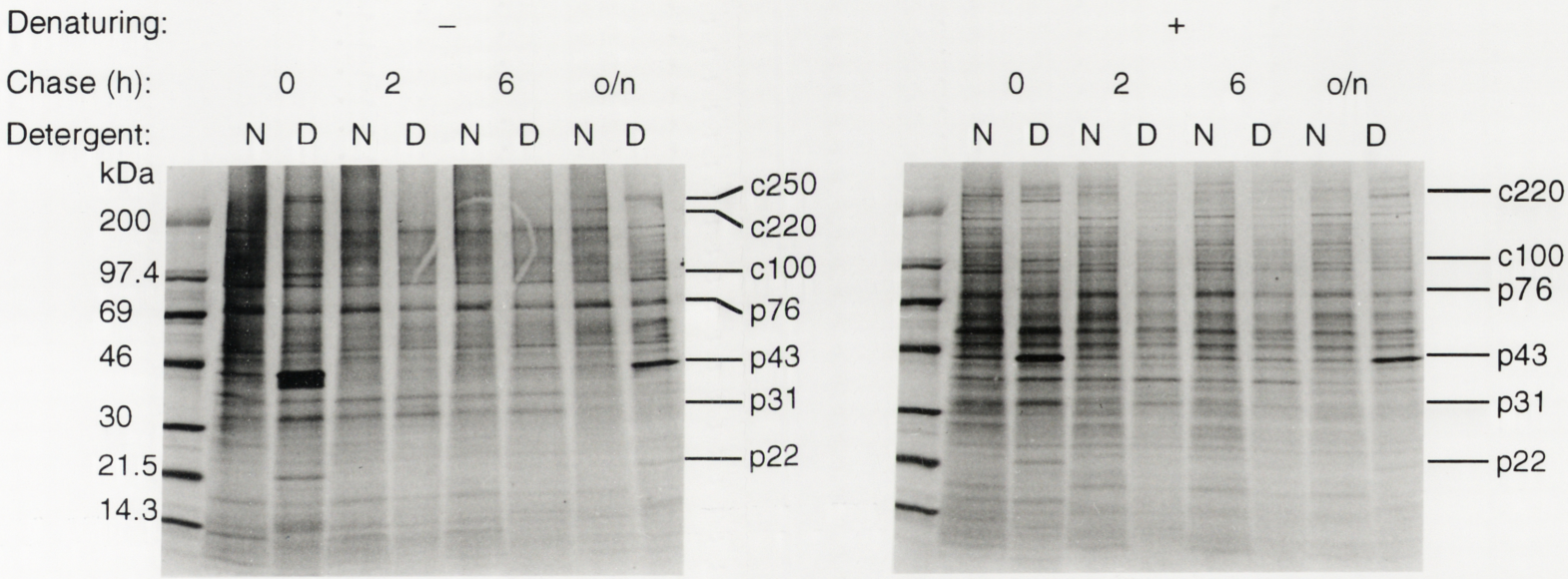


Fig. 3.4a **b**


Denaturing:
–
+
Chase (h):
0
2
6
o/n
Detergent:
N D N D N D N D
kDa
200
97.4
69
46
30
21.5
14.3
c250
c220
c100
p76
p43
p31
p22

NP40 and digitonin lysates, which suggests that H2-M is dissociated from large complexes. Another notable difference between the two detergents was that a higher concentration of the 76kDa band in NP40 lysis was seen at all chase times with respect to that obtained from digitonin lysis.

Collectively, these results show that there are some significant differences in the molecules precipitated when NP40 and digitonin are used for lysis and subsequent immunoprecipitation.

3.4. The effect of pH on immunoprecipitation of MHC class II-associated molecules

Proteins and protein complexes may be precipitated differentially in an immunoprecipitation experiment at different pH depending on their solubilities and complex stabilities. A protein or a protein complex which is recognized by a given antibody at a certain pH may be precipitated less or not at all precipitated at another pH. A protein and its complexes may be insoluble at a certain pH or they may be aggregated and not recognized on the protein gel. Additionally, protein complexes may be sensitive to pH and dissociate during all lysis thereby abrogating co-precipitation.

Since MHC class II-associated protein complexes, especially H2-M associated protein complexes, were co-precipitated in digitonin more effectively than in NP40 (see Section 3.3 for details), the effect of pH on co-precipitation of MHC class II-associated molecules by immunoprecipitations was observed employing digitonin lysis only. Proteins were metabolically labelled with [^{35}S]-methionine for 2h and then the cells were lysed in digitonin lysis buffer at various acidities, namely pH7, pH6, pH5 and pH4. I-A^{d}, Ii and H2-M associated molecules were then precipitated at the same pH as that of which the lysis was performed and they were

analyzed with non-denaturing sample extraction only. Methods for these immunoprecipitations have been described in detail in Section 2.4.1.

Briefly, A20 cells were washed and depleted of intracellular methionine by incubation in methionine-free RPMI 1640 medium twice for 30 minutes. Cells were collected and incubated with [^{35}S]-methionine in a fresh RPMI 1640 medium for 2 hours at 37°C. Cells were washed with cold PBS and divided equally into 4 portions. Each portion of the cells was lysed in 1% digitonin lysis buffer at pH7, 6, 5 or 4 for 15 minutes at 4°C. The lysates were spun at high speed to remove cellular debris. Lysate supernatants were then precleared with normal rabbit serum in the presence of protein A sepharose CL-4B 4 times for 3 hours at 4°C. Beads were spun briefly and the precleared cell lysate supernatants were immunoprecipitated for 2 hours at 4°C with MKD.6, P4H5 or K553 with protein A sepharose-6MB for I-A^d, Ii or H2-M precipitation, respectively. The concentration of antibodies used for immunoprecipitation is detailed in Table 2.2. Immunoprecipitates were washed gently 4 times in the corresponding lysis buffer, and an additional 2 washes were performed in digitonin-free citrate-phosphate buffer. Precipitates were eluted in a Tris-Tricine gel loading buffer by incubation for 25 minutes at 37°C before analysis with 10-20% Tris-Tricine gradient gel electrophoresis. Proteins separated by electrophoresis in SDS were transferred onto nitrocellulose membranes and exposed to an autoradiography film.

The results of the immunoprecipitations with anti-I-A^d antibody MKD.6 at different acidities extracted under non-denaturing conditions are shown in Fig 3.5a and summarized in Table 3.2. There were clear differences in the intensity of bands arising from molecules precipitated at different pH. One striking example of this phenomenon is that p90 is precipitated at pH5 only. Similarly, MHC class II α and β monomers were visible clearly in the precipitations at pH7 and pH6, however, they were reduced dramatically at pH5 and pH4. A molecule at the size of the

Table 3.2. Summary of molecules precipitated according to Fig. 3.5a, b and c.

Figure	Fig. 3.5a				Fig. 3.5b				Fig. 3.5c				expected/ precised molecule
Antibody used	MKD.6				P4H5				K553				
Cell lysis pH	7	6	5	4	7	6	5	4	7	6	5	4	
Mole-cule size (kDa)	120	120	120		120	120	120		120				
			90				90						
	80	80	80	80	80*	80	80	(80)	80	80	80	80	
	60*	60			60*	60	60	60	60*				Ii p31 dimer
	57	(55/7)	55/7	55	57	(55)	55	55					$I.A^d$ dimer
	50				50				50*	50	(50)		H2-M dimer
			43	43*	43	43	43	43*		43	43	43	
	33*	33	33	33	33*	33	33	33	33*	33	(33)	(33)	$I.A^d\alpha$
	31*	31	31	31	31*	31*	31*	31	31				Ii p31
									<31	<31	<31	(<31)	H2-Ma
	28*	28	28	(28)	28*	28	28						$I.A^d\beta$
		25				25	25	(25)		(25)	(25)	(25)	Ii fragment
				22				22		(22)	(22)	(22)	Ii fragment
									20	20	20		Ii fragment
	10	10				10	10	(10)	(10)	10	10		Ii fragment
			3	3			3	3			3	(3)	CLIP

* : strong intensity of the molecule in the same size.
( ) : weak band
< : lower size

compact class II dimer (c55) appeared more clearly at lower pH, *i.e.* pH5 and pH4. The strong intensity of Ii-31 at pH7 was much reduced at pH6 while, by contrast, p25 and p10 (SLIP) Ii fragments cleaved from p31 were precipitated most effectively at this pH. In addition, an intense band at 60kDa in the precipitation at pH7 was identified as a dimer of Ii-31, because dimeric Ii was identified at the same size by Western analysis in A20 cell lysates at the same pH in a subsequent experiment (Fig. 6.2a). Furthermore, Roche *et al.* (1991) have shown also that dimers of Ii at the same size co-purify with class II molecules from B-LCL Swei cells. The strong intensity of the dimeric form of Ii-31 at pH7 was reduced at lower pHs in parallel to the reduced intensity of monomers of Ii-31. Also, this decrease of concentration in Ii-31 and dimers of Ii-31 $(Ii\text{-}31)_2$ at lower pH had the same pattern as the decrease of concentration in Ii-31 and $(Ii\text{-}31)_2$ in the MKD.6 immunoprecipitation of pulse chase experiments at pH7 (see Fig. 3.1a).

An intense band at 43kDa was precipitated at pH4 only with MKD.6 but this molecule was not identified. Also, an I-$A^d\alpha$ size band, 33kDa, and a LIP size band, 22kDa, were precipitated dominantly at pH4 but an I-$A^d\beta$ size band was not seen at this pH although this immunoprecipitation was performed with the anti-I-A^d antibody, MKD.6. This indicates that I-$A^d\beta$ may not be soluble at pH4. Additionally, all molecules precipitated at pH5 were generally less abundant than molecules precipitated at the other pHs. This might have been caused by the loss of some precipitate during the immunoprecipitation process.

Protein bands obtained by immunoprecipitation with the anti-Ii antibody P4H5 at different pH are shown in Fig. 3.5b. Generally speaking, a similar pattern to the bands precipitated with MKD.6 is apparent in that there were also clear differences in the molecules precipitated at different pH.

Ii-31 was precipitated by P4H5 at pH7 the most and the intensity of the band was reduced dramatically at lower pHs, also $(Ii\text{-}31)_2$ was precipitated in the same

manner as precipitation of Ii-31 at different pH, the most at pH7 and much reduced at lower pH. However, bands at 25kDa and 10kDa were seen predominantly at pH6 and pH5 and weakly at pH4 in the anti-Ii immunoprecipitations.

I-$A^d\alpha$ was co-precipitated with anti-Ii immunoreactivity at each pH investigated although the intensity of this band was reduced progressively as the pH was lowered. Also, I-$A^d\beta$ was observed, migrating as a band below Ii-31, strongly at pH7, weakly at 6 & 5 and even more weakly at pH4. The greater reduction in the amount of I-$A^d\beta$ precipitated at low pH compared to that of I-$A^d\alpha$ may indicate the relative insolubility of I-$A^d\beta$ at low pH, as observed in the MKD.6 immunoprecipitation above. Furthermore, a band at the size of a floppy form of I-A^d dimers (60kDa) was seen at pH7 and disappeared at pH6, 5 and 4 while a band corresponding to the compact form of the molecules at 55kDa was apparent clearly at pH5 and even more clearly at pH4.

A band at 80kDa was precipitated by P4H5 at all pH conditions and the band at 90kDa was precipitated with the same antibody at only pH5, as was the case in the immunoprecipitaton with MKD.6

[^{35}S]-methionine autoradiographs obtained from immunoprecipitations with the anti-H2-M antibody at different pH are presented in Fig 3.5c. Overall, the intensity of bands precipitated was much lower (refer to Section 3.2 for reasons) compared with the intensity of bands precipitated by MKD.6 or P4H5 even though more cells (2-fold) were used for these immunoprecipitations. The polyclonal anti-serum K553 appeared to precipitate lower amounts of material at lower pH, in contrast to MKD.6 and P4H5.

A band at 80kDa was seen at all acidities in the anti-H2-M precipitates as was the case in anti-I-A^d and anti-Ii immunoprecipitates. The intensity of the band at 50kDa, the size of the H2-M dimer, was greatest at pH7, was much weaker at pH6 and disappeared as the pH was lowered. Distinct bands at 60 and 31kDa were

seen at pH7 only (present faintly at lower pH) and these molecules were confirmed as being dimeric and monomeric forms of Ii-31, respectively, by Western analysis in later experiments (see Fig 4.4a for details). A band which migrated very close to Ii-31 is apparent at pH7 and it is present in all precipitates with a progressive decrease in the intensity of the band at low pH. The identity of this band was confirmed subsequently as Ma by Western blotting with the anti-Ma antibody #104 (see Fig. 4.3 for details). Bands which are proteolytic Ii fragments, such as p25, p21 and p10, were seen mostly at pH6 and pH5. A CLIP size molecule (3kDa) was precipitated also in the anti-H2-M precipitates at pH5 as was the case for both the anti-Ii and anti-I-A^d precipitates. The high molecular weight band c90, which was seen at pH5 both in the anti-Ii and anti-I-A^d precipitations, was not seen in the precipitates induced by K553. Interestingly, the band at I-A^d dimer size which was seen clearly, especially at low pH, both in the anti-Ii and anti-I-A^d precipitates was not seen in anti-H2-M precipitates, however, the band at I-$A^d\alpha$ size was seen in these precipitates.

Overall, I-A^d, Ii and H2-M were precipitated along with their associated molecules (as co-precipitates) in a pH dependent manner and the respective complexes of the primary target proteins showed similar pH-dependent stability. In each immumoprecipitation, Ii-31 was precipitated most abundantly at pH7, the bands at the size of Ii intermediates, 25kDa and 10kDa, were precipitated principally at pH6 and 5, and the band corresponding to CLIP was most apparent in precipitations at pH5. A high molecular weight band at 90kDa was observed in anti-I-A^d and anti-Ii precipitates at pH5 only and this molecule may not be able to be detected in traditional experiments conducted at pH7. The variation in the proteins and complexes precipitated by these antibodies as a function of pH was rather marked. This along with the bases for identification and any implication

related to the stability of protein complexes participating in the MHC class II pathway at different pH is discussed in the next section.

3.5. Discussion

The results presented here have shown that the selection of both the detergent and the pH for an immunoprecipitation has a significant impact upon the precipitation of MHC class II-associated molecules.

Immunoprecipitation of I-A^d class II-associated molecules in the pulse-chase experiment in the detergent NP40 at pH7 using MKD.6 (Fig 3.1a) has shown a similar pattern of bands to that obtained by other workers (Weenink *et al.*, 1996) under the same conditions. Firstly, 60-55kDa class II αβ dimers appeared more abundantly after the 6h and overnight chase periods. The absence of I-A^d αβ dimers on SDS-PAGE gel at the shorter chase times is explained by the fact that I-A^d dimers which are newly synthesized and bound with Ii in the ER are not stable in SDS (Busch *et al.*, 1996), dissociating to the monomers These I-A^d dimers, which are bound to Ii, are transported along the endocytic pathway and localized in MIIC by virtue of the endosomal targeting signal at the N-terminus of Ii (Viville *et al.*, 1993; Zhong *et al.*, 1996). During transport along the endocytic pathway, Ii is degraded by proteases in the endosomal compartments after 2-3h (Blum and Cresswell, 1988; Maric *et al.*, 1994), leaving the final Ii fragments associated with class II molecules (CLIP) in the peptide binding groove of I-A^d molecule (Rudensky *et al.*, 1991). After 3-4h, in MIIC, CLIP is dissociated from I-A^d class II molecules with co-operation by H2-M (mouse homologue of HLA-DM) and antigenic peptides (or self-peptides) are loaded in the groove of the I-A^d molecule (Neefjes *et al.*, 1990; Sherman *et al.*, 1996). The I-A^d class II molecules bound with a peptide are transported to the cell surface to present the peptide to $CD4^+$ T cells after 4h (Wubbolts *et al.*, 1996). The I-A^d αβ dimers which are bound with

peptides in MIIC are formed more compactly than those without peptides and they are stable in SDS (Lindstedt *et al.*, 1995). In spite of the absence of exogenous antigenic peptides in intact A20 cells, I-A^d $\alpha\beta$ dimers are still able to form SDS stable complexes on SDS-PAGE gel by binding endogenous peptides (but the amount is much lower than in the presence of exogenous antigenic peptides). Thus, the formation of I-$A^d\alpha\beta$ dimers apparent at longer chase periods in this pulse-chase experiment is consistent with the current understanding that generally 4 to 6h are required after the molecules were synthesized in the ER for complete class II antigen presentation to have occured on the cell surface (Neefjes *et al.*, 1990). The intensity of I-$A^d\alpha$ and β monomers including I-A^d dimers precipitated with MKD.6 at 0h was much lower than 2h chase time point. This may be due to the fact that insufficient time has elapsed for the cells to have completed the synthesis and post-translational modification of these proteins labelled with [^{35}S].

Secondly, Ii-31 was precipitated by MKD.6 most abundantly after the [^{35}S]-methionine pulse and its concentration was reduced dramatically at the 2h chase time point. Concomitantly, the Ii fragments p25 and p10 were seen to appear at the 2h chase time and the 31kDa band of intact Ii was not seen at the 6h chase time-point or thereafter. This result and the fact that no intact [^{35}S]-labelled Ii is present at the time by which temporally co-synthesized class II molecules are present at the cell surface is clearly indicative of Ii degradation in endosomal compartments (Nguyen *et al.*, 1989; Riese *et al.*, 1996). This decrease of intensity in Ii-31 bands at longer chase times is paralleled by that of Ii dimer bands at 60kDa ((Ii-31)$_2$, Fig. 3.1a). The decreased intensity of (Ii-31)$_2$ at 2h chase in this co-immunoprecipitation was consistent with the explanation offered by Marks *et al.* (1990) that the dimeric form of Ii is an intermediate between trimer and monomer forms and that it is not observed after loss of the cytoplasmic domain by proteolysis in the endosomal compartments.

In the anti-Ii immunoprecipitations (Fig. 3.1b), the relative abundance of Ii-31 and $(Ii\text{-}31)_2$ reflected Ii degradation at longer chase times also, as was observed in the MKD.6 immunoprecipitations. The dimeric form of Ii was seen clearly at 0h and 2h chase times under non-denaturing conditions and at the 0h chase time under denaturing conditions (see Fig 3.1b). The Ii fragments p22 and p10, which were co-precipitated with $I\text{-}A^d$ (see Fig 3.1a), were not precipitated by P4H5 in this set of immunoprecipitations. This might be explained by the fact that p22 and p10 are MHC class II-associated Ii fragments and these proteins could be co-precipitated with $I\text{-}A^d$ class II molecules more readily than being directly precipitated by the anti-Ii antibody, P4H5. Also, Ii is synthesized in molar excess over class II molecules (Cresswell, 1992) and, in the absence of dynamic exchange, only a small amount of $I\text{-}A^d$-associated Ii fragments would exist relative to the total Ii population. The film may require a very long exposure time to see these fragments, resulting in overexposure of $[^{35}S]$-Ii-31 which gives a very strong signal on the film.

In the immunoprecipitation with anti-H2-M antibody K553 (Fig. 3.1c), Ma was seen to migrate very close to Ii-31 under both non-denaturing and denaturing conditions (see also Fig. 5.3a). In the report of Karlsson *et al.* (1994), Ma was found to run just above Ii-31, but Ma seems to run just below the Ii-31 band in these experiments. This assignment was made according to the differences in the degradation pattern of these two bands at different time chases. Ii-31 is degraded by proteases in the acidic environment of endosomal compartments after leaving the ER (Nguyen *et al.*, 1989; Harding *et al.*, 1991; Reyes *et al.*, 1991; Maric *et al.*, 1994; Riese *et al.*, 1996) but H2-M dimers are transported to and accumulate in the major antigen processing compartments, MIIC, without degradation after synthesis in the ER (Sanderson *et al.*, 1994). This difference was clearly reflected also in Fig. 3.5c which showed clear differences in the degradation state of these

two molecules which also precipitated selectively at different pH and their relative size on the Tris-Tricine SDS-PAGE gel indicated that Ii was running higher than H2-M. The difference in the relative electrophoretic mobility of H2-M between these immunoprecipitations and the work of Karlsson *et al.* (1994) might be due to the employment of different cell lines or different protein gel systems. In this work, A20 cells and 10-20% Tris-Tricine gradient gels were used and B10.M splenocytes and 7.5-12.5% Tris-glycine polyacrylamide gels were used in immunoprecipitations of Karlsson *et al.* (1994). This molecule (H2-M) was co-precipitated by MKD.6 most abundantly after 6h chase under non-denaturing conditions (Fig.3.1c) which is consistent with the observations of Copier *et al.*(1996) that DM accumulates in MIIC, where peptide loading occurs by a process which involves physical association of MHC class II molecules and DM.

The [^{35}S]-methionine pulse-chase experiment is thus a powerful tool for the investigation of the kinetics of the synthesis, maturation and degradation of proteins participating in the MHC class II pathway.

The pulse-chase experiments were repeated with digitonin and NP40. Perusal of Fig. 3.2, 3.3 and 3.4 reveals that there were clear differences in the molecules precipitated depending upon the chaotropic strength of the detergent used. Generally, large molecular complexes were dominant in precipitations using digitonin lysis extracted under non-denaturing conditions, such as c120 and c60 in the anti-I-A^{d} precipitations and c250 and c100 in the anti-H2-M precipitations (see Table 3.1). By contrast, lower molecular weight proteins including p28, p27, p25 and p18 were precipitated more effectively in NP40 lysis. Exceptionally, K553 (Fig. 3.4) precipitated a high molecular weight complex, c220, in NP40 lysis which was evident under non-denaturing analytical conditions but was not apparent when digitonin was used. Also, the same antibody precipitated a small protein, p23, in digitonin lysis which was visualized under both non-denaturing and denaturing

conditions but which was not evident in precipitations in NP40. The most striking example of a detergent related effect is the presence of protein, p43, in the digitonin lysate at 0h and after the overnight chase period in the anti-H2-M immunoprecipitations (Fig. 3.4). This band has not been identified yet and identification of this molecule may implicate a new molecule involved in I-A^d class II antigen presentation.

Generally, the results presented here suggest that aggregates or complexes of the molecules which are involved in the class II pathway are precipitated more effectively using digitonin lysis. Presumably, this is because they are not especially stable and are easily dissociated in a strong detergent like NP40. Also, small molecules appeared to be precipitated more effectively in NP40 than in digitonin lysis. These molecules may be more soluble and, hence, are detected more readily in the strong detergent NP40 than in the comparatively weak detergent, digitonin, or they may be dissociation products generated in the harsher conditions of the strong detergent. The detergent effect was especially significant in the anti-H2-M precipitations where in more co-precipitated bands were obtained with digitonin than with NP40 lysis (see Fig 3.4a or Table 3.1). This suggests that H2-M has a tendency to be weakly associated with other molecules in cells and that these intracellular associations may not be able to be detected in an immunoprecipitation coupled with strong detergent lysis.

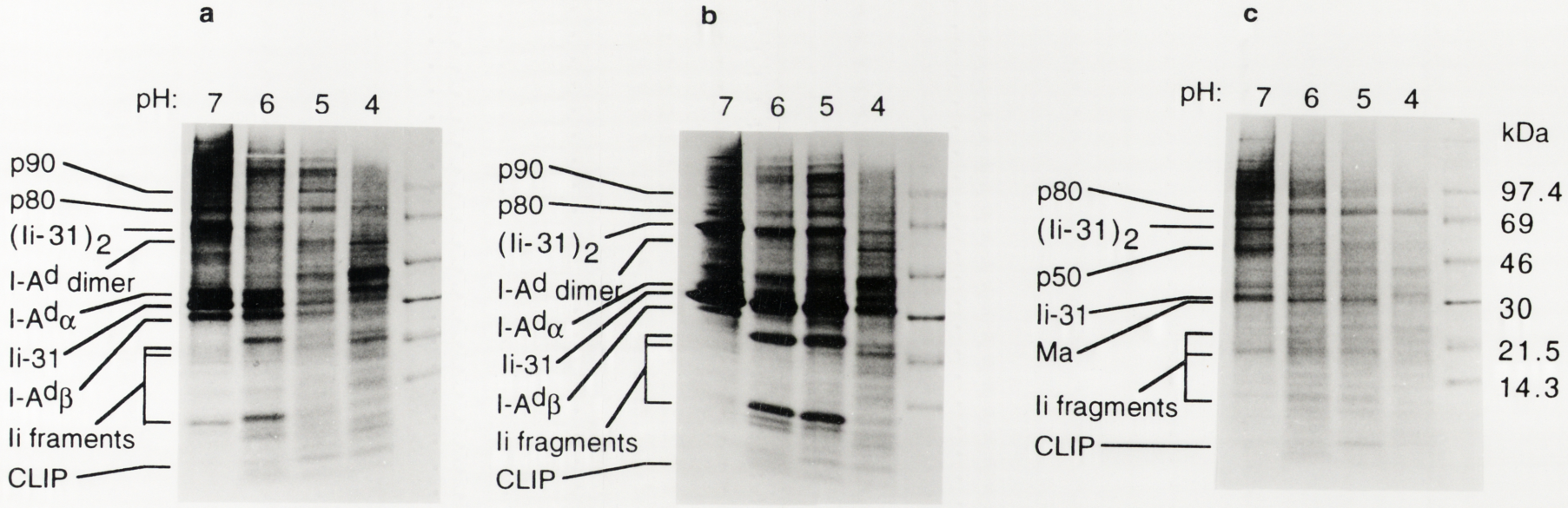
For immunoprecipitations seeking to achieve biochemical analysis of MHC class II-associated molecules, the choice of detergent is very important in order to effect the co-precipitation of molecules participating in MHC class II antigen presentation. Each molecular association has a different stability and solubility properties and different detergents have both different abilities to lyse cells and solubilize molecules. Most immunoprecipitations to date have been done in NP40 lysis, which is effective for immunoprecipitation by virtue of its property to give

strong lysis of cell membranes and effective solubilization of proteins, however, some molecules which are weakly associated may not be co-precipitated due to dissociation in NP40. In a previous report by Sanderson *et al.* (1996), it has been shown that the DM/DR association is weak and that this complex is precipitated effectively only in weak detergent (digitonin) lysis. Therefore, comparison of detergent ability is a prerequisite to recover molecules involved in the class II pathway maximally.

The acidity at which cell lysis and immunoprecipitation was performed had a profound effect upon the profile of protein bands obtained (Fig. 3.5). The results from the analysis of detergent dependency revealed that more bands appeared to be associated with H2-M when precipitated at pH7 using digitonin lysis (Fig. 3.4). For this reason, cell lysis and immunoprecipitation of molecules involved in the I-A^d class II antigen presentation was performed at different acidities using digitonin lysis only, under non-denaturing conditions of sample extraction.

The rationale behind this experiment is to determine whether protein complexes are differently precipitated as a function of pH. Antibody recognition and solubility of protein complexes may vary as a function of pH. Additionally, complexes may disintegrate after prolonged exposure to an acidity at which they are unstable. For example, Ii fragments precipitated with P4H5 (such as p25 and p10) at pH6 only, neither at pH7 nor at pH5 and pH4 (Fig. 3.5b) which probably reflects pH-dependent antibody recognition.

The appearance of the strong 55kDa band at low pH (pH5 and 4) in the anti-I-A^d immunoprecipitations showed that the peptide-loaded SDS-stable I-A^d dimers have their greatest stability at low pH. In contrast, the loss of intensity in 33kDa and 28kDa bands at pH5 and 4 showed that monomeric forms of I-A^d α and β were not readily visualized in the electropherograms at low pH. Especially, the decrease in the amount in I-$A^d\beta$ was greater than I-$A^d\alpha$ as the pH was lower,


a
pH: 7 6 5 4
p90
p80
(Ii-31)2
I-Ad dimer
I-Adα
Ii-31
I-Adβ
Ii frament
CLIP
b
7 6 5 4
p90
p80
(Ii-31)2
I-Ad dimer
I-Adα
Ii-31
I-Adβ
Ii fragments
CLIP
c
pH: 7 6 5 4
kDa
97.4
69
46
30
21.5
14.3
p80
(Ii-31)2
p50
Ii-31
Ma
Ii fragments
CLIP

which may indicate lower relative solubility of I-$A^d\beta$ than I-$A^d\alpha$ at low pH. The antibody MKD.6 recognizes the β-chain in the intact I-A^d dimer, therefore the β-chain must be present originally in the immunoprecipitation in same abundance as the α-chain.

Ii-31 and $(Ii\text{-}31)_2$ were co-precipitated with I-A^d class II molecules most abundantly at pH7, which shows a maximum stability of association of these molecules with I-A^d at this pH. In contrast, the Ii fragments, p25 and p10, were co-precipitated with I-A^d at pH6 only, neither at pH7 nor pH5 or pH4 as mentioned above. These I-A^d/p25 and I-A^d/p10 complexes appear to have maximal stability at pH6. These complexes might be aggregated as large insoluble complexes or unstable and thus dissociated before precipitation at other pH conditions and, hence, were not visible on the gel. Alternatively, the antibody, MKD.6, recognizes those complexes selectively at pH6 only in the pool of various protein complexes.

A small protein band at 3kDa was co-precipitated with I-A^d at pH5 and 4 and this size of molecule corresponds to the size of CLIP identified in the immunoprecipitations of DR3 in T2.DR3 cells described by Riberdy *et al.* (1994). It is expected that these final fragments of Ii (CLIP) that are bound in I-A^d class II molecules are generated by further degradation of p25 and p10. The co-precipitation of CLIP with I-A^d at pH5 and pH4 shows the stability of I-A^d/CLIP complexes at low pH. The proteins p31, p25, p10 and p3 are all I-A^d-associated molecules since these were co-precipitated with I-A^d class II molecules by the anti-I-A^d antibody MKD.6. The selective co-precipitation of CLIP with I-A^d at pH5 is consistent with the finding of Bangia and Watts (1995) that CLIP binding to I-E class II molecules was maximal at pH5.5 in their binding assay.

The protein bands obtained by immunoprecipitation with P4H5 (Fig. 3.5b) had a pH-dependent pattern similar to the molecules precipitated by MKD.6 as a

function of pH. The proteins Ii-31 and $(Ii\text{-}31)_2$ were precipitated most abundantly at pH7 showing the greatest stability of the Ii/I-A^d association at this pH as in the anti-I-A^d immunoprecipitations (Fig. 3.5a). Also, a band at the size of the I-A^d dimer, appearing at lower pHs indicated high stability of this molecule at low pH, in contrast, the loss of intensity in the bands at I-A^d α (33kDa) and β (28kDa) sizes at lower pHs showed a low stability of I-A^d monomers at these pHs as observed in the MKD.6 immunoprecipitations at different pH. Furthermore, I-$A^d\beta$ was seen to be less soluble than I-$A^d\alpha$ at low pH as in the MKD.6 immunoprecipitations of I-$A^d\alpha\beta$ heterodimers. The Ii fragments, p25 and p10, were precipitated abundantly at pH6 and 5 in the P4H5 anti-Ii immunoprecipitations although these molecules were co-precipitated with I-A^d at pH6 only in MKD.6 immunoprecipitations. This may be explained by the different selectivity of the antibody, P4H5, at different pH. Additionally, the intensity of bands, such as c60, p43 and p31 had less variation at different pH in the P4H5 immunoprecipitations than in the MKD.6 precipitations. This result might have been caused by the different selectivity of the different antibodies for these complexes at the same pH or these molecules (for p43) may be primary precipitants for P4H5.

The immunoprecipitations of H2-M and associated molecules at different pH were characterized by weak intensity bands and high background, presumably because the antibody K553 is unpurified anti-serum and H2-M expression in the cell is relatively low (Schafer *et al.*, 1996). The band corresponding to Ma migrates very close to Ii-31 and is present at pH7, however this Ma band was present clearly at pH6 but at reduced abundance, while the Ii-31 band had disappeared at this pH, indicating that the Ii-31/H2-M association may not be stable at pH6. Furthermore, the co-precipitation of Ii-31 with Ma at pH7 suggests the possibility of their association in the cell under neutral conditions. The dimeric form of H2-M was precipitated principally at pH7, suggesting that H2-M readily

dissociates into Ma and Mb in the presence of SDS at low pH. Other molecules precipitated or co-precipitated differentially in a pH-dependent manner in K553 immuunoprecipitations showed a similar pattern to the molecules precipitated at different pH in the anti-I-A^d immunoprecipitations. These are a band at 80kDa at each pH used, the strong intensity of Ii-31 and (Ii-31)2 at pH7 and Ii fragments (p25, p22, p10 and p3) at low pH. Also, as seen in the anti-I-A^d and anti-Ii immunoprecipitations, high molecular weight bands were precipitated at pH7 much more abundantly than at other pHs in the H2-M precipitates. However, the bands at 90kDa and I-A^d dimer size precipitated by MKD.6 and P4H5 are not seen in the K553 immunoprecipitations.

In the immunoprecipitations at different pH, bands at 120kDa and 80kDa were precipitated with all 3 antibodies at pH7 and the intensity of this band was reduced when the pH was lower. Other molecules which were precipitated differentially as a function of pH were a 90kDa protein which was evident at pH5 only and a 43kDa protein most abundant at pH4 in the anti-I-A^d and anti-Ii precipitations. The band at a 90kDa protein may be a good example of a protein from a pH sensitive complex which is not detected using the traditional method of immunoprecipitation at pH7. Also, the precipitation of p43 maximally at pH4 may indicate another aspect of the selectivity conferred by pH in immunoprecipitation. Therefore, immunoprecipitation over a range of pH may be a good method by which to analyze the pH-sensitivity of the molecular complexes present in cell lysates.

Previously, Ii intermediates, LIP and SLIP, and DM were co-purified with DR3 from leupeptin treated .114 cells (Schafer *et al.*, 1996). However, CLIP association with DM (or H2-M) has not been detected yet. Further analysis will be required to confirm the H2-M/CLIP association at low pH. Two protein bands, 90kDa and 43kDa, may represent new molecules involved in the I-A^d class II

pathway, hence, identification of these molecules may be also necessary to discover further molecular processes involved in the MHC class II antigen presentation.

The pH dependency of co-precipitation observed in Fig 3.5 conveys the clear impression that class II-associated molecular complexes reside in the intracellular compartments at their most stable pH. For example, intact Ii is known to associate with MHC class II molecules after synthesis in the ER (Busch *et al.*, 1996) and (Ii-31)$_2$ and Ii-31 were co-precipitated with I-A^d class II molecules at the pH of the ER, pH7, most abundantly (Fig. 3.5a) in this experiment. Also, Ii intermediates degraded by proteases in endosomal compartments are associated with MHC class II molecules (Nguyen *et al.*, 1989) and bands at 25kDa and 10kDa which are the same sizes as these Ii intermediates were co-precipitated with I-A^d at the pH of the endosomal compartments, pH6 (Fig. 3.5a).

In conclusion, cell lysis and immunoprecipitation in detergents with different chaotropic strength precipitated different molecules depending in part on the stability of the macromolecular complexes. Also, MHC class II-associated molecules were precipitated differentially in a pH-dependent manner depending in part on the stability of these specific complexes. The complexes appeared to be maximally stable at a pH which reflects their intracellular origin. Therefore, detergent and pH are key factors which affect results of immunoprecipitations of I-A^d class II-associated molecules.

The protein H2-M is not seen abundantly in all autoradiographs and, consequently, these may not be very informative for investigation the interactions of this protein. However, it appears that H2-M is associated with Ii at pH7 in digitonin lysis (Fig. 3.5c). Additionally, H2-M is associated with I-A^d at pH7 (Fig. 3.5a). Therefore, it is possible that all three molecules, I-A^d, Ii & H2-M, may be associated at pH7. To characterize further the association of H2-M with I-A^d and Ii at different pH, immunoprecipitation based on the optimal conditions were

conducted followed by Western analysis. These experiments will be presented in the following Chapter.

Chapter 4

4. Identification of molecules co-precipitated with H2-M at different pH

4.1. Introduction

Although HLA-DM (and by analogy H2-M) has been described as a peptide "editor" (Denzin and Cresswell, 1995) residing in the major antigen processing compartments, MIIC, functioning by catalyzing the exchange of peptides bound to class II molecules (Kropshofer *et al.*, 1997), the localization of DM in the cell and the nature of its association with other molecules in the class II pathway are still unclear and presently under debate. DM is known to be associated with class II dimers in the MIIC (Sanderson *et al.*, 1996) but its localization is not restricted to these compartments (Pierre *et al.*, 1996), which allows the possibility of DM association with other molecules in the endocytic pathway. Previously, LIP and SLIP, but not intact Ii-31, along with DM have been co-purified with DR from leupeptin-treated .114 cells by Schafer *et al.* (1996). However, the association of Ii-31 and H2-M related proteins has been observed by co-precipitation in digitonin lysis of Ii-31Δ and H2-MΔ transfected HeLa cells (Lindstedt *et al.*, 1995). The proteins Ii-31Δ and H2-MΔ are mutants of Ii-31 and H2-M, both of which have a deletion of endosomal targeting motifs. Therefore, the location and extent of the association of Ii with DM in the cell and the manner of their interaction remains unclear.

The experiments described in the previous Chapter relied upon [^{35}S]-methionine protein labelling visualized with autoradiographs which provided limited information on the association of H2-M with I-A^d or Ii in the murine MHC class II pathway. Immunoprecipitations with MKD.6 and P4H5 (anti-I-A^d and anti-Ii, respectively) with analysis by [^{35}S] autoradiography allowed investigation of the pH and detergent dependent effects on co-precipitation with I-A^d and Ii.

However, the polyclonal anti-H2-M antibody, K553, extracted H2-M from cell lysates in immunoprecipitations with a high non-specific background because this antibody is an unpurified anti-serum. Additionally, DM is expressed at significantly lower levels than class II molecules in .114 cells (1:23 ratio in the cell, Schafer *et al.*, 1996). As a result, the [^{35}S] intensity of the bands relating to H2-M on the autoradiography film (see Fig. 3.5c) were not distinct compared to the [^{35}S] intensity of I-A^{d} and Ii related species (see Fig. 3.5a) although larger numbers of cells (double) were used for K553 precipitations. Consequently, investigation of the co-precipitation of H2-M with I-A^{d} or Ii in a pH dependent manner (see Chapter 3), required the identification of these molecules by Western analysis.

In an attempt to co-precipitate H2-M-associated molecules optimally, immunoprecipitations have been performed on digitonin lysates of A20 cells at a range of pHs corresponding to that found along the MHC class II pathway, namely pH7, 6, 5 and 4. Identification of the co-precipitated molecules has been pursued by means of Western analysis with the relevant antibodies.

4.2. H2-M identification in cell lysates

In order to study H2-M intracellular associations by co-immunoprecipitation at different pH, it was considered to be necessary to verify the identification of H2-M in cell lysates over the same pH range. This identification of H2-M was achieved by Western analysis with the antibodies #104 (anti-Ma) or 1B9A (anti-Mb).

The general method for the direct identification of H2-M from cell lysates by immunoblotting was described in Section 2.5. Briefly, A20 cells were washed in cold PBS and lysed in 1% digitonin (for Ma detection) or 1% NP40 (for Mb detection) lysis buffer, at pH7 or pH5, containing protease inhibitors (see Section 2.4.1) for 20 minutes at 4°C. The cell lysates were centrifuged to remove cellular debris and lysate supernatants were divided into 2 equal portions to be analysed

under non-denaturing and denaturing conditions. One portion of the lysates was mixed with either Tris-Tricine or Tris-glycine gel loading buffer for non-denatured samples and the other portion of the lysates was denatured by boiling for 5 minutes in the same loading buffer containing 2.5% 2-ME. Both non-denatured and denatured cell lysates were loaded onto either a 10-20% Tris-Tricine gradient gel or a 12% Tris-glycine gel. Proteins were separated by gel electrophoresis and then electroblotted onto nitrocellulose membranes. These membranes were used for Western analysis to identify Ma or Mb. After blocking non-specific protein binding sites on the membrane, the membrane was incubated with either the antibody #104 or 1B9A for either Ma or Mb identification, respectively. Membranes washed in PBS-T were again incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit IgG-HRP for the primary antibody #104 and anti-rat IgG-HRP for 1B9A. After further washing, Ma and Mb immuno-reactivity was detected on the membrane by means of the ECL protein detection system. A negative control experiment was performed using the same procedure except that the primary antibody was omitted in each case prior to blotting with the secondary antibody.

As Fig 4.1 shows, Ma was identified clearly both at pH7 and pH5 in cell lysates, indicating that the outcome of lysis is independent of the pH used and detection is possible irrespective of the denaturing conditions. Ma has 261 amino acids and is anticipated to have a relative molecular mass of 33-35kDa with 2 N-linked glycosylations, as explained in Section 1.4.1. From the membrane immunoanalysis, Ma mobility was almost identical to Ii-31, as described in Section 3.2. This result accords with that of Karlsson *et al.* (1994) who found that Ii-31 and Ma were seen at almost the same position on a gel after immunoprecipitation from B10.M splenocytes with K553 (anti-H2-M antibody).

Fig. 4.1

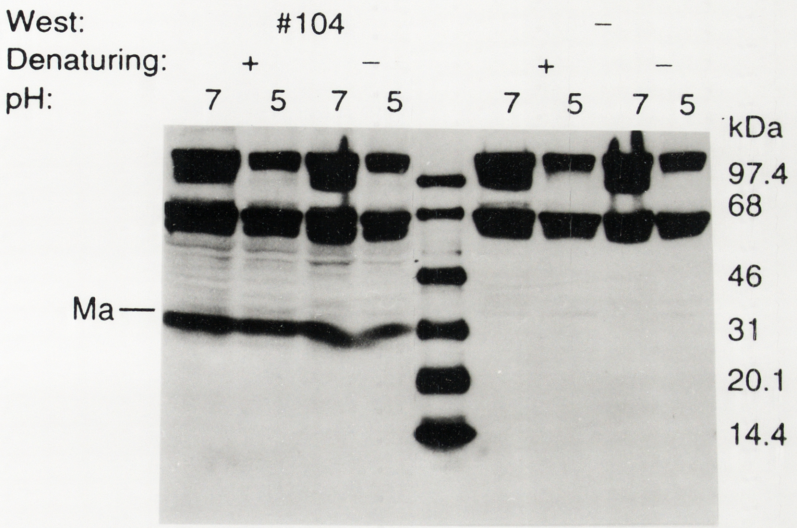
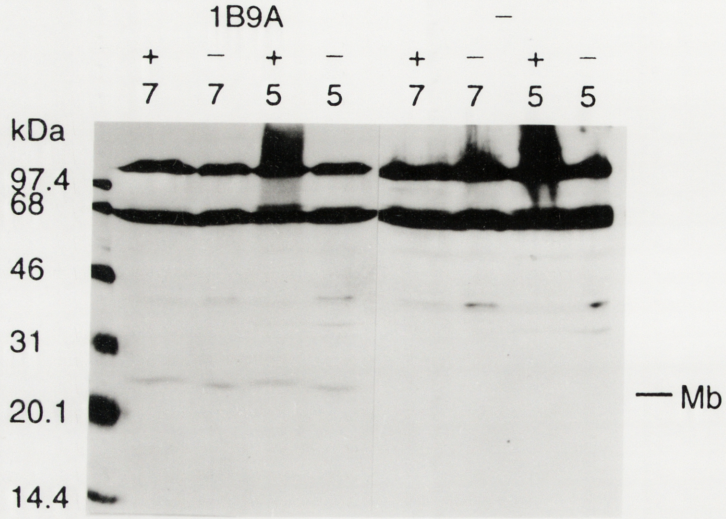

West:
#104
–
Denaturing:
+
–
+
–
pH:
7
5
7
5
7
5
7
5
kDa
97.4
68
46
31
20.1
14.4
Ma

Fig. 4.2


1B9A
–
+
–
+
–
+
–
+
–
7
7
5
5
7
7
5
5
kDa
97.4
68
46
31
20.1
14.4
Mb

The results of immunoblotting an electropherogram of A20 cell lysates with 1B9A (Fig 4.2) showed Mb weakly but clearly at both pH7 and pH5, again establishing the independence of detection from the pH used for lysis and the sample extraction conditions. According to Karlsson *et al.* (1994), Mb has 2 isoforms: Mb1 and Mb2, with quite a number of sequence differences between 2 genes and Mb1 has a N-linked glycosylation site which is not present in Mb2. As a consequence, Mb1 size is 31-33kDa MW and Mb2 size is 27kDa MW although they both have the same number of amino acids (see Fig 1.7). The size of Mb on the membrane indicates that the Mb in A20 cells is the Mb2 isoform.

4.3. Dependence on pH of co-precipitation of H2-M with Ii

To investigate the pH dependence of the association of Ii-31 with H2-M at acidities corresponding to those in intracellular compartments, H2-M-associated molecules were co-precipitated from lysates performed at different pH (pH7, 6, 5 & 4) by immunoprecipitation with K553. Having confirmed the presence of Ma in the anti-H2-M precipitates by immunoblotting with #104, Ii presence was then observed in the same anti-H2-M precipitates by Western analysis with In-1. To confirm this result in the reciprocal experiment, Ma presence was observed in anti-Ii (P4H5) precipitates by immunoblotting with #104.

The immunoprecipitations were performed using the method described in Section 3.4 for K553 immunoprecipitations over a range of pH with the exception that both non-denaturing and denaturing extractions from the beads were analysed by SDS-PAGE.

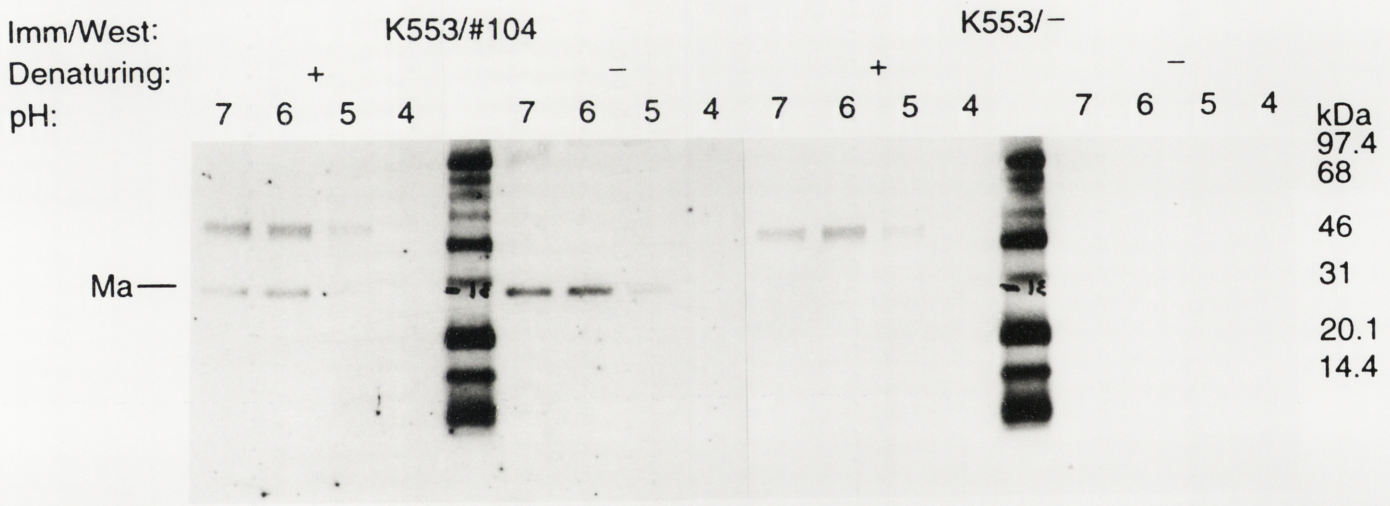
Briefly, A20 cells (see Fig. 3.5 legend for cell numbers) were depleted of intracellular methionine for 1 hour and labelled with [^{35}S]-methionine (1mCi/100 million cells) for 2 hours. Cells were then divided into four portions and lysed in 1% digitonin lysis buffer at pH7, 6, 5 or 4 containing protease inhibitors (see

Fig. 4.3. Identification of H2-M in A20 cells at different pH by immunoprecipitation.

The procedure and conditions for immunoprecipitation with K553 from A20 cell lysates are the same as in Fig. 3.5c. Briefly, A20 cells were lysed in 1% digitonin lysis buffer at different pH, pH7, 6, 5 and 4, and cell lysates were immunoprecipitated with K553 to precipitate H2-M and associated molecules. The precipitates were extracted from the sepharose beads under non-denaturing and denaturing conditions and resolved by 10-20% Tris-Tricine gradient gel electrophoresis followed by electroblotting onto nitrocellulose membranes. Then, detection of Ma in the K553 immunoprecipitates was performed by Western analysis for H2-M, as described for the identification of Ma in the cell lysates (see legend for Fig. 4.1) including a negative control experiment.

Abbreviations: West, Western blot: Imm, immunoprecipitation: +, denatured: -, non-denatured.

Fig. 4.3


Imm/West:
K553/#104
K553/−
Denaturing:
+
−
+
−
pH:
7 6 5 4 7 6 5 4 7 6 5 4 7 6 5 4
kDa
97.4
68
46
31
20.1
14.4
Ma—

Section 2.4.1) for 15 minutes at 4°C. The precleared cell lysates were immunoprecipitated with K553 for 2 hours at 4°C in the presence of protein A sepharose-6MB. Precipitates were eluted sequentially in Tris-Tricine gel loading buffer without boiling or reduction for the non-denatured samples. Non-denatured extracts were transferred into other tubes and the beads were extracted for a second time by boiling for 5 minutes in the same loading buffer containing 2.5% 2-ME for the denatured samples. Each of both the non-denatured and denatured samples were divided into 2 portions for different Western analysis and loaded on 10-20% Tris-Tricine gels before electrophoresis. The electrophoresed proteins were transferred onto nitrocellulose membranes for Western analysis. The Western blotting for Ma or Ii identification was performed by following the method described in Section 4.2. After blocking non-specific protein binding sites, the membranes were blotted with either #104 or In-1 as the primary detection antibody for Ma or Ii identification, respectively. To provide a negative control for this experiment, exactly the same process was performed as above except without the addition of the primary antibodies.

Examination of the Western analysis for Ma in the anti-H2-M precipitates confirmed the presence of this protein, as a band at 31kDa in both non-denatured and denatured bead extracts over a range of different acidities (Fig 4.3). In fact, the Ma from the anti-H2-M precipitates was detected at each pH at which immunoprecipitation was performed except pH4.

Immunoanalysis of the membrane from the anti-H2-M (K553) precipitates with In-1 (Fig. 4.4) shows that Ii-31 was co-precipitated from H2-M precipitation essentially at pH7 only, although H2-M was present in the same precipitates from pH7 to pH5 (Fig. 4.3) and Ii-31 was present at pH7 and pH6 in the anti-Ii precipitates (data not shown). This result suggests that the H2-M/Ii-31 interaction

H2-M association with Ii at different pH.

Fig. 4.4. Co-precipitation of Ii from anti-H2-M immunoprecipitates.

Detection of Ii from anti-H2-M precipitates was performed by the same procedure of immunoprecipitation and Western blot used in Fig. 4.3. However, the primary antibody In-1 and secondary antibody anti-rat IgG-HRP were used for the identification of Ii in H2-M precipitates. For a negative control of identification of Ii, the primary antibody was omitted.

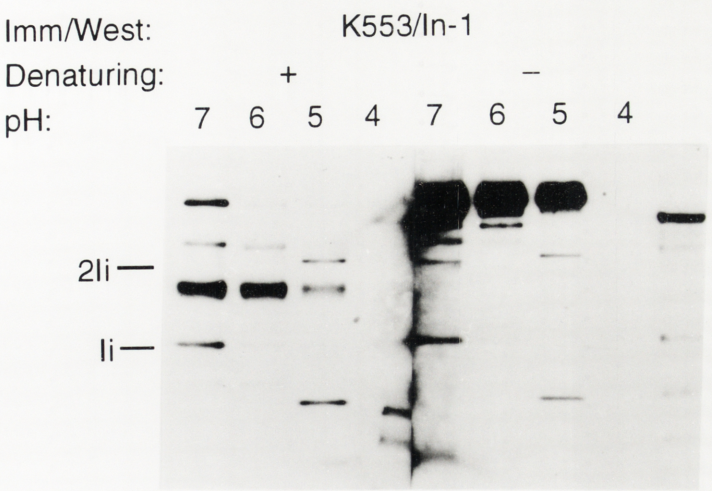
Abbreviations: Imm, immunoprecipitation: West: Western blot.

Fig. 4.5. Co-precipitation of H2-M from anti-Ii immunoprecipitates.

Detection of H2-M from anti-Ii precipitates was performed using the same method and conditions used in Fig. 4.4 with the exception that P4H5 was used to precipitate Ii and associated molecules from A20 cell lysates. Also, #104 and anti-rabbit IgG-HRP were used as primary and secondary antibodies for the identification of H2-M from anti-Ii-precipitates.

Abbreviations: Imm, immunoprecipitation: West: Western blot.

Fig. 4.4a



b

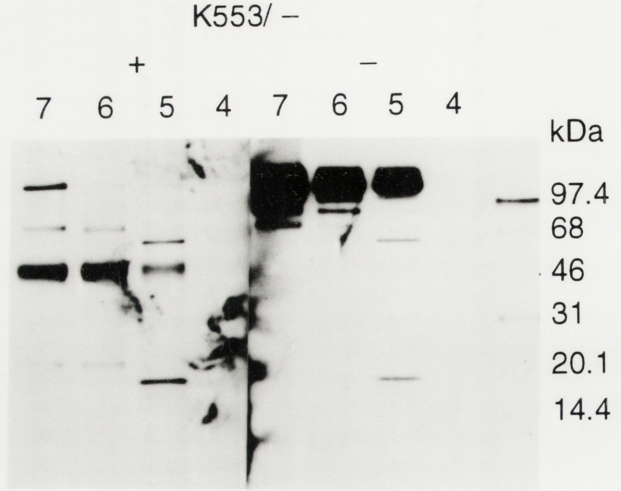
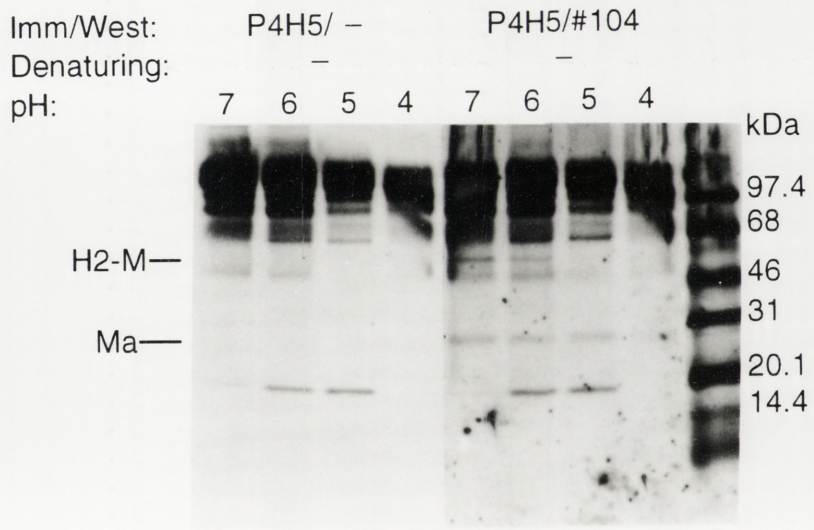


Fig. 4.5



is the most stable at pH7. It also possible that there is a pH dependent selectivity for these complexes in the immunoprecipitation.

To corroborate the pH-dependence of the H2-M/Ii-31 interaction, identification of H2-M as a co-precipitant in anti-Ii precipitates has been performed. The methodology for the immunoprecipitation and Western analysis are the same as for the Ii detection from K553 precipitates as described above; with the exceptions that P4H5 was used to precipitate Ii and associated molecules and #104 was used for detection of Ma co-precipitation from P4H5 primary precipitates. Additionally, samples were analysed using non-denaturing bead extraction only for this experiment, since this analysis is a conformation of the H2-M/Ii-31 association at pH7 and H2-M dimers (which are associated with Ii) are expected to be seen at this pH under non-denaturing conditions.

In Fig 4.5, Ma was seen clearly at pH7 to pH5 in anti-Ii precipitates and, additionally, a band at the size of H2-M dimers, 50kDa, were seen at pH7 and pH6, indicating that H2-M may associate with Ii under a range of acidities corresponding to those found in the endocytic pathway. The apparent discrepancy between the results of these two experiments, which are Ii-31 detection primarily at pH7 in anti-H2-M precipitates and H2-M detection at pH7 to pH5 in anti-Ii precipitates, could arise from one or more of several sources, including H2-M being associated with Ii fragments which lack the recognition site of the In-1 antibody (residues 2 to17 (Mehringer *et al.*, 1991)). This will be discussed later, in Section 4.5.

Overall, it is likely that the Ii-immunoreactivity which was associated with H2-M at pH6 and pH5 derives from H2-M-associated Ii fragments because, at pH6 and pH5, intact Ii-31 in P4H5 anti-Ii precipitates was not detected in anti-H2-M precipitates (see Fig.4.4). Also, in Fig. 3.5c, while Ii-31 is absent, bands at Ii

fragment sizes including p25, p23, p20, p10 and p3 have been shown by autoradiography of K553 immunoprecipitates at pH6, 5 and 4.

4.4. Dependence on pH of co-precipitation of H2-M with I-A^d class II molecules

In order to examine the pH dependence of the association of H2-M with I-A^d class II molecules, H2-M and associated molecules were immunoprecipitated with K553 over a range of pH (pH7, 6, 5 & 4) by the method described in Section 4.3. Subsequently, the presence of I-A^d was then determined in the anti-H2-M precipitates by Western analysis. Samples were extracted from the beads under denaturing conditions only, because I-A^d dimer bands (between 50-60kDa) were seen more clearly under denaturing conditions than non-denaturing conditions (see Fig. 3.1c and 3.4). The membrane was probed with bio-MKD.6 together with bio-M5/114, followed by streptavidin-HRP and developed by ECL to identify I-A^d dimers and monomers in the H2-M precipitates.

As evident from Fig. 4.6, I-A^d dimers were detected in anti-H2-M precipitates at each pH and the abundance of the I-A^d which was associated with H2-M was apparently the greatest at pH5. This suggested that the H2-M/I-A^d association may be the most stable at pH5.

To verify the association of I-A^d with H2-M, the reciprocal co-precipitation of H2-M with I-A^d was examined. Detection of H2-M from anti-I-A^d immunoprecipitations was performed in the same manner and using the same conditions described above for the detection of I-A^d in the anti-H2-M precipitates, except that MKD.6 was used to precipitate I-A^d and associated molecules and #104 was used in the immunoblotting for Ma identification in the anti-I-A^d precipitates. Again, samples were analyzed under non-denaturing conditions only because the pattern of protein bands at low molecular weight (under 70kDa)

H2-M association with I-A^d class II molecules at different pH.

Fig. 4.6. Co-precipitation of I-A^d from anti-H2-M immunoprecipitates.

Immunoprecipitation of H2-M and associated molecules from A20 cell lysates was performed as described in Fig. 4.4. Western analysis using bio-MKD.6 and bio-M5/114 in combination was performed for the identification of I-A^d in the anti-H2-M precipitates. The method of Western analysis for I-A^d identification is described in Section 2.5 and the concentration of the antibodies used in this Western analysis is detailed in Table 2.2.2 and 2.2.3.

Abbreviations: Imm, immunoprecipitation: West: Western blot: $\alpha\beta$, I-$A^d\alpha\beta$.

Fig. 4.7. Co-precipitation of H2-M from anti-I-A^d immunoprecipitates.

Immunoprecipitation of I-A^d and associated molecules from cell lysates was performed as described in the legend for Fig. 4.6 with the exception that antibody MKD.6 was used for this immunoprecipitation. Then, identification of H2-M in the anti-I-A^d precipitates was carried out using the same procedure and conditions of Western analysis described in Fig. 4.3, including the negative control of the analysis.

Abbreviations: Imm, immunoprecipitation: West: Western blot.

Fig. 4.6

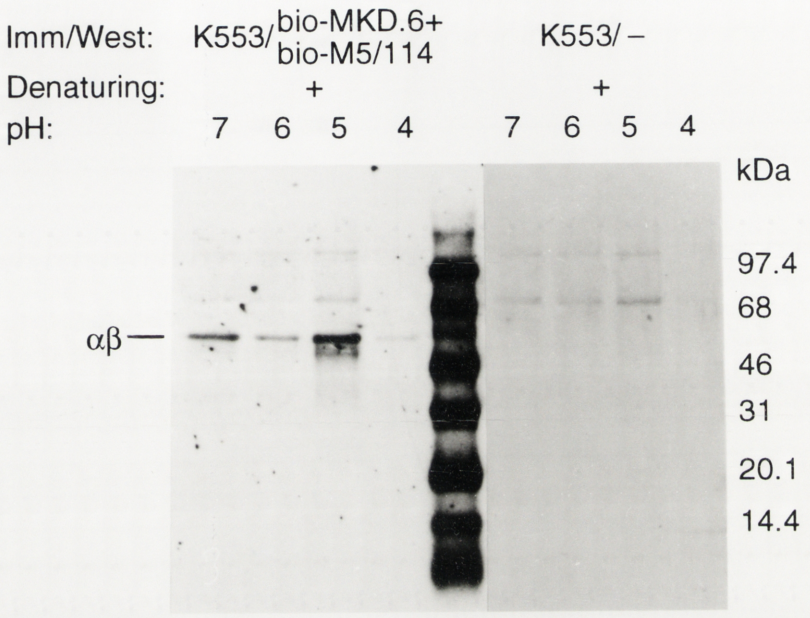
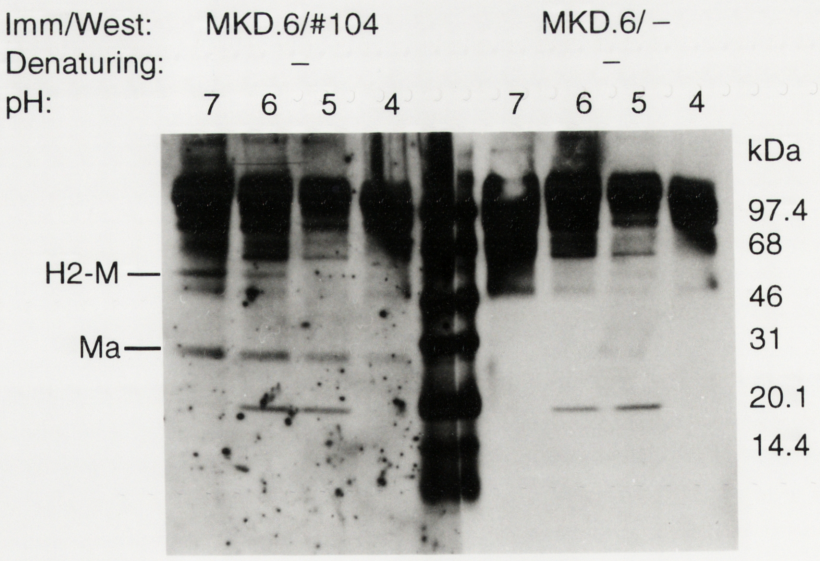

Imm/West:
K553/bio-MKD.6+ bio-M5/114
K553/–
Denaturing:
+
+
pH:
7 6 5 4
7 6 5 4
kDa
97.4
68
46
31
20.1
14.4
αβ

Fig.4.7


Imm/West:
MKD.6/#104
MKD.6/–
Denaturing:
–
–
pH:
7 6 5 4
7 6 5 4
kDa
97.4
68
46
31
20.1
14.4
H2-M
Ma

extracted under non-denaturing and denaturing conditions from anti-I-A^d precipitates was similar (see Fig. 3.1a), also H2-M dimers which may be associated with I-A^d class II molecules were expected to persist under non-denaturing extraction conditions.

As shown in Fig 4.7, H2-M was clearly visible at pH7 and pH6 and Ma was detected at each pH used in the anti-I-A^d immunoprecipitates. This result is consistent with the previous finding that I-A^d was co-precipitated with H2-M at all acidities investigated (Fig. 4.6).

4.5. Discussion

Detection of H2-M in A20 cell lysates by immunoblotting (Fig. 4.1 & 4.2) revealed that both Ma and Mb were detected in lysis at pH7 and pH5. This indicates that H2-M is apparently soluble in a manner independent of acidity. Furthermore, Ma was detected following digitonin lysis and Mb was detected following NP40 lysis, indicating efficient immunoprecipitation of H2-M independent of the detergent used. The apparently lower yield of Mb with respect to Ma in the cell lysates may reflect different sensitivity of these antibodies in the Western analysis. Since, the identification of H2-M in precipitates with #104 had a higher sensitivity for Ma compared to that of 1B9A for detection of Mb, the antibody #104 was used for the detection of the H2-M co-precipitation in anti-Ii or anti-I-A^d precipitations.

The anti-H2-M precipitates (Fig. 4.3) were most efficient at pH7 and 6, while Ma was precipitated in much lower yields at pH5 and it was not precipitated at all at pH4. This indicates that the ability of the antibody, K553, to precipitate H2-M is lower at low pH.

Western analysis provided a powerful tool to investigate co-immunoprecipitation of H2-M with I-A^d and Ii. The co-precipitation of these

molecules was pH dependent. In the investigation of the association of Ii with H2-M at a range of acidities (Fig. 4.4 & 4.5), intact Ii-31 was co-precipitated with H2-M at pH7 only but H2-M was co-precipitated with Ii immunoreactivity from pH7 to pH5, suggesting that H2-M associates with Ii-31 at pH7 and possibly with Ii fragments at pH6 and pH5. The stable association of H2-M with Ii-31 at pH7 allows the possibility that these molecules may be associated after being synthesized in the ER. This suggestion is consistent with the finding of Karlsson *et al.* (1994) that Ii-31 was co-precipitated with H2-M immediately after the [^{35}S]-methionine from pulse B10.M splenocytes in a pulse-chase experiment. Furthermore, the stable association of H2-M with Ii fragments at pH6 and pH5 allows the possibility of their association in endosomal compartments of similar pH. If this pH stability represents an origin in endosomal compartments, it may be possible that H2-M associates with Ii fragments, cleaved by proteases in the endocytic pathway, which are not recognised by In-1. The Ii fragment bands, including p25 and p10, were not visible at pH6 and pH5 in the anti-H2-M precipitates by immunoanalysis with In-1 (Fig 4.4) but were visualised by autoradiography (see Fig. 3.5c). Also, the association of H2-M with Ii fragments has been precedented by the results of Schafer *et al.* (1996) who detected Ii fragments, such as LIP and SLIP, with DMB in purified DR3 from leupeptin treated .114 cells.

The result that H2-M and I-A^{d} were co-immunoprecipitated after digitonin lysis of A20 cells over the range pH7 to pH4 but maximally at pH5 (Fig. 4.6 & 4.7) demonstrates that this association is sufficiently stable such that it has potential to persist from the ER throughout the endocytic pathway to the MIIC. In contrast, Sanderson *et al.* (1996) have reported that the association of HLA-DR and DM in Raji cells was detected by immunoprecipitation from digitonin lysis at pH5 only, not in NP40 lysis at pH5 or in either detergent at pH7. The apparent disagreement

of the results may arise from such factors as sequence differences between I-A^d and DR class II molecules, H2-M and DM, other species specific differences and differentially efficient immunoprecipitation and Western analysis. It should be noted that K553 appeared to precipitate H2-M inefficiently at lower pH (fig. 4.3) and, consequently, the pH-dependency of I-A^d co-precipitation may be more marked than is immediately appeared from perusal of Fig. 4.6. Thus, the strongest co-precipitation of I-A^d and H2-M in digitonin lysis of A20 cells at pH5 in this experiment is consistent with the result that HLA-DR and DM were co-precipitated with each other only in digitonin lysis of Raji cells at pH5. A direct interaction of purified DM with DR3 at a low pH (pH5) has been shown by Ullrich *et al.* (1997) but association of DM with MHC class II molecules at any other pH has not been reported previously.

Perusal of Fig. 4.7 also demonstrates co-precipitation of H2-M with I-A^d over a range of pH. While this I-A^d/H2-M association was most stable at pH5 in the anti-H2-M immunoprecipitations, this was not apparent in the reciprocal experiment. This may be caused by the reduced ability of MKD.6 to precipitate molecules at low pH or it is possible that there may be more than one population of H2-M in association with I-A^d. For example, H2-M may associate with both Ii and I-A^d in the ER (less stable at pH7) or I-A^d only in MIIC after Ii is degraded (more stable at pH5).

Previous studies have shown that I-A^d class II molecules associate with intact Ii in the ER (Busch *et al.*, 1996) and with Ii fragments in the endocytic pathway (Blum and Cresswell, 1988; Maric *et al.*, 1994). Results from Fig. 3.5a also showed that I-A^d and Ii-31 appeared to form an association which is most stable at pH7, the pH of the ER, but that the association of I-A^d with Ii fragments was more stable at lower pH (Fig. 3.5a). Taken together the pH dependence of these co-immunoprecipitations raises the possibility that all three molecules, H2-M, Ii and I-

A^d, may be associated in the ER at pH7. Dissociation of Ii-31 would be expected at lower pH in the endosomal compartments, while H2-M and I-A^d may still associate with Ii intermediates in the more acidic conditions of the endocytic pathway to the MIIC. Thus, H2-M may associate with both Ii and I-A^d in the class II pathway and the nature of these association may be different in different intracellular compartments. In order to investigate these hypotheses, pulse-chase experiments were designed in double immunoprecipitation of I-A^d, Ii and H2-M. The re-precipitated molecules were shown by autoradiography and then they were identified by Western analysis. These will be presented in the following Chapter.

Chapter 5

5. H2-M intracellular association by pulse-chase experiments and double immunoprecipitation

5.1. Introduction

As discussed in the preceding Chapter, the co-immunoprecipitation of H2-M, Ii and I-A^d from A20 cells has led to the hypothesis that all three molecules may associate in different ways at different stages in the class II pathway. From the pH dependency of these co-precipitations, there is the possibility that the nature of their association differs in different intracellular compartments which are at different pH. However, the possibility of their association and the pattern of any such association in the class II pathway needs to be confirmed.

In a pulse-chase experiment, as described in Section 3.2, intracellular methionine is depleted in cells and the depleted methionine is replaced with [^{35}S]-labelled methionine for a short period, *e.g.* 30 minutes. During this period, protein molecules synthesized in the ER of the cells are labelled with [^{35}S]-methionine. After the [^{35}S]-labelling period, the cells are incubated with an excess of [^{32}S]-methionine in the medium. The radioisotopically labelled [^{35}S]-proteins are present predominantly in the ER at the end of pulse period. Proteins involved in the MHC class II antigen presentation have endosomal targeting signals in their sequence (minor splice variants of Ii have an ER retention signal also, *e.g.* Ii p35 and p43 (Wolf, P. R. and Ploegh, H. L., 1995). By virtue of these signals, they are transported to post-Golgi vesicles by 2h, MIIC by 4h and to the cell surface by 4h after synthesis (Neefjes *et al.*, 1990). After a defined time period of incubation of the cells in the unlabelled medium following the [^{35}S]-pulse, the cells are lysed, and a population of molecules and the associated molecular complexes are immunoprecipitated by an antibody against the target molecule. The immunoprecipitated molecules are separated by SDS-PAGE and electroblotted

onto a cellulose membrane. The [^{35}S] labelled molecules among total protein molecules in an immunoprecipitation are visualised by virtue of the incorporated [^{35}S] upon exposure of SDS-PAGE gels or electroblots to autoradiography film. The labelled molecules precipitated at a certain time in the chase are presumed to originate from certain intracellular compartments, as discussed above.

The specific identity of radioactive bands may be confirmed by immunoblotting with a relevant antibody. However, changes in the concentration of radiolabelled molecules with respect to the chase time may not be distinguished by Western analysis since the rate of protein synthesis and degradation is assumed to be constant under normal conditions. Hence, the concentration of a given protein, as visible in the Western blot, is constant independent of time after the [^{35}S] pulse at which the molecule was precipitated.

In preliminary experiments, the primary immunoprecipitations gave a high background (Fig.3.1 - 3.5), especially, the anti-H2-M precipitations (Fig.3.5c). The electrophoretic mobility of Ii and Ma was found to be very similar (see Fig.4.4 and 5) and it was hard to resolve and visualize these proteins separately by autoradiography alone. By re-immunoprecipitation of a molecule with the pertinent antibody from a primary immunoprecipitation, the background on the gel is reduced and co-precipitations may be defined more specifically.

For these reasons, double immunoprecipitation against specific molecules at different times in an [^{35}S]-methionine pulse-chase experiment was adopted as a strategy to confirm the association of the three molecules (I-A^{d}, Ii and H2-M) in the class II pathway.

The general strategy of the pulse-chase experiment for each time point of the primary immunoprecipitation was performed using the method described in Section 3.2. After the primary immunoprecipitation, the precipitated molecules on the sepharose beads were re-solubilized in 1% SDS for 2h at 37°C, then another

immunoprecipitation was performed once again in NP40 lysis buffer, which now contains 0.1% SDS, with a different antibody. The beads were washed in the buffer corresponding to the NP40 lysis buffer and the re-precipitated molecules were eluted under denaturing conditions only, due to the limiting amounts of the re-precipitated molecules. The eluates from the second immunoprecipitation were resolved by SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The membranes were analyzed by autoradiography and Western analysis. For immunoblotting more than one molecule on the same membrane, the membrane was stripped as described in Section 5.2 before analysis for a second molecule. Otherwise, different lanes of the protein gel on the same membrane were used for the identification of different molecules.

5.2. Intracellular H2-M/Ii association by re-immunoprecipitation

The observation that the co-precipitation of Ii-31 with H2-M occurred only at pH7 (Fig. 4.4) and that the co-precipitation of H2-M with Ii fragments (Fig. 4.5) occured at pH6 and pH5 suggested different origins for the associated complex within the endocytic pathway. To confirm this hypothesis, the H2-M/Ii association was re-examined by coupling the [^{35}S] pulse-chase experiment with re-immunoprecipitation of the molecules from primary precipitates. The proteins present in the re-immunoprecipitation were investigated by immunoanalysis with the appropriate antibody. Also, the relative stability of the complex was probed by using two detergents with different chaotropic strengths, NP40 and digitonin, to lyse cells and to immunoprecipitate relevant targets.

The detailed method for the re-immunoprecipitation of Ii or H2-M from anti-H2-M or anti-Ii primary precipitates, respectively, at different times of chase was described in Section 2.4.2. Briefly, intracellular methionine in A20 cells (see Fig. 5.1-3 legend for cell numbers) was depleted and then the cells were pulsed with

[^{35}S]-methionine (1mCi/100 million cells) for 30 min. The labelled molecules were chased for 0, 3 and 6h or overnight and cells were then lysed in 1% digitonin or 1% NP40 lysis buffer (see Section 2.2 for the composition of the buffers) containing protease inhibitors (see Section 2.4.1). Lysates were precleared and immunoprecipitated with K553 or P4H5 for H2-M or Ii and their associated molecules, respectively. After elution of the precipitates from protein A-conjugated beads in 1% SDS for 2h at 37°C, either H2-M or Ii was then re-precipitated with K553 or P4H5 from Ii or H2-M primary precipitates, respectively, in 1% NP40 lysis buffer. These re-precipitates were reduced in Tris-Tricine loading buffer containing 2.5% 2-ME and denatured by boiling for 5 minutes before analysis by 10-20% Tris-Tricine gradient gel electrophoresis followed by electroblotting onto nitrocellulose membranes. After exposure of the membranes to an autoradiography film, each track of the protein gel on the membrane was cut for Western analysis for different molecules To identify re-precipitated molecules, each strip from different membranes was immunoblotted with #104 or In-1 for Ma or Ii identification in H2-M or Ii re-precipitates, respectively. A negative control experiment was performed on the other strip of the membrane using the same procedure except that the primary antibody was omitted

As shown in Fig 5.1a, the P4H5 re-immunoprecipitation from K553 primary precipitates in digitonin lysis clearly showed a band at 31kDa immediately after the [^{35}S]-methionine pulse (0h) which showed substantial degradation into smaller molecules, principally at 28kDa, by the 6h and longer chase periods. The larger band was shown to have Ii-31 immunoreactivity with In-1 but the degraded band at 28kDa was not identified by this antibody (Fig. 5.1b). Thus, the Ii-31 which was re-precipitated from anti-H2-M primary precipitates corresponds to that Ii which was bound with H2-M sufficiently tightly so that it was not dissociated in NP40 or 1% SDS.

Fig. 5.1-3. H2-M is intracellularly associated with Ii and this association is strong enough not to be dissociated in NP40.

Association of H2-M with Ii was detected by re-immunoprecipitation of H2-M or Ii from anti-Ii or anti-H2-M primary precipitates, respectively, at different chase times. The method and conditions of the re-immunoprecipitation are described in Section 2.4.2. Briefly, A20 cells (3 to 6 million cells/well of protein gel) were depleted of intracellular methionine for 1h. Cells were then pulsed with [^{35}S]-methionine for 30 min and chased for 0, 3, 6h or overnight. Chased cells were lysed in 1% digitonin (**5.1** and **5.2**) or 1% NP40 (**3**) lysis buffer at pH7 containing protease inhibitors for 20 min at 4°C. After preclearing, H2-M or Ii and associated molecules were immunoprecipitated with K553 (**5.1** and **5.3**) or P4H5 (**5.2**) in the presence of protein A sepharose-6MB beads for 2 h at 4°C. Sepharose beads were washed and precipitates were eluted from beads in 1% SDS for 2 h at 37°C. Ii or H2-M was re-precipitated with P4H5 (**5.1** and **5.3**) or K553 (**5.2**) in 1% NP40 lysis buffer from anti-H2-M or anti-Ii primary precipitates, respectively. The re-precipitates were denatured by boiling for 5 minutes in a Tris-Tricine gel loading buffer with 2.5% 2-ME before analysis by 10-20% Tris-Tricine gradient gel electrophoresis. After gel electrophoresis, the re-precipitated molecules were electroblotted onto nitrocellulose membranes and then exposed to an autoradiography film (**a**) before immunoblotting with an antibody In-1 (**5.1b** and **5.3b**) or #104 (**5.2b**). Western analysis to identify Ii or H2-M in anti-Ii or anti-H2-M re-precipitates has been performed by the method described in Section 2.5. The same procedure without applying primary antibody has been followed to serve us a negative control for each Western analysis.

Abbreviations: Im, immunoprecipitation: re, re-immunoprecipitation: West, Western blot: o/n, overnight: NC, negative control.

Fig. 5.1a **b**

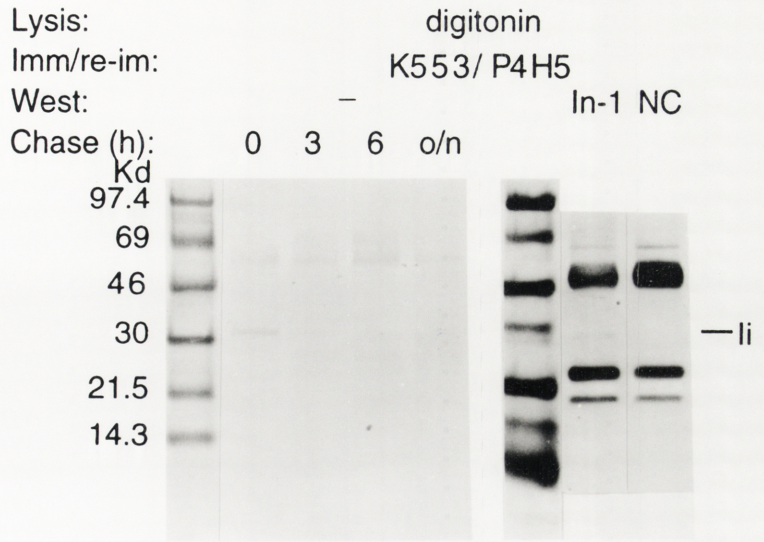


Fig. 5.2a **b**

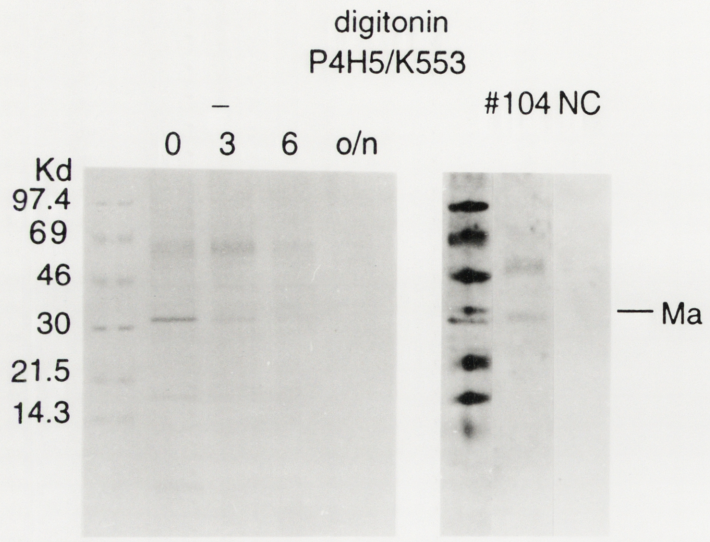
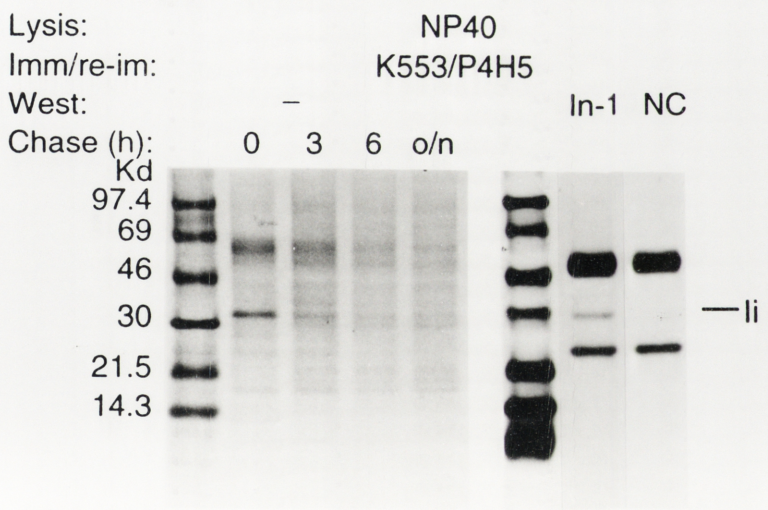


Fig. 5.3a **b**



To confirm the observation that intact Ii-31 was re-precipitated from anti-H2-M precipitates due to association, the converse experiment has been executed, in which P4H5 primary precipitation in digitonin lysis was performed at different times after a 30 minute [^{35}S]-methionine pulse followed by K553 re-precipitation in NP40 (Fig. 5.2a). Once again, a protein band was re-precipitated at 31kDa, immediately after pulse (0h). The intensity of the band was reduced after the 3h chase period and it persisted as a band present faintly at 6h chase. This precipitation did not show the degraded band at 28kDa which appeared in the K553 primary and P4H5 re-immunoprecipitations as the chase time was longer (Fig. 5.1a), suggesting that the band was primarily comprised of Ma and not Ii-31. Immunoblotting with antibody #104 confirmed the presence of Ma in this band (Fig 5.2b). Therefore, H2-M was re-precipitated from anti-Ii precipitates at least until 6h chase period as well as early after pulse, which indicates association of H2-M with Ii-derived species immunoreactive to P4H5 in the endocytic pathway in addition to the ER. The electrophoretic mobilities of Ii-31 and Ma are very similar on the protein gel (see Section 4.3) and Ii-31 was also re-precipitated from anti-H2-M primary precipitates at 0h chase (see Fig. 5.1). Hence, it was suspected that this band (Fig. 5.2a, 0h, 31kDa) might also contain Ii-31 especially because this protein is methionine rich (14 methionines) and the band had a high intensity of ^{35}S, especially at 0h chase. After stripping the membrane for 30 minutes at 50°C in the stripping buffer (100mM 2-ME, 2% SDS and 62.5mM Tris-HCl, pH6.7), this band was immunoblotted again with In-1 for the identification of Ii. The band was observed to have Ii-31 immunoreactivity also, as expected (data not shown).

Next, to investigate the nature of the Ii/H2-M association, a strong detergent NP40 was used for both primary immunoprecipitation and re-precipitation. The re-immunoprecipitation of Ii from anti-H2-M primary precipitates with A20 cells was carried out by exactly the same process as described in Fig.5.1a, with the

exception that cells were lysed in 1% NP40 lysis buffer for the primary immunoprecipitations. The re-precipitated molecules were analysed by 10-20% Tris-Tricine gel electrophoresis (Fig. 5.3a) followed by Western analysis with In-1 to identify Ii (Fig. 5.3b). Interestingly, Ii-31 was re-precipitated at 0 h chase from anti-H2-M primary precipitates of NP40 lysis as shown in Fig. 5.1a.

In summary, H2-M associates with Ii after their synthesis in ER and their association is strong enough not to be dissociated during NP40 lysis and the immunoprecipitated complex is stable to 1% SDS. This association is detectable until at least 3h after the [^{35}S]-methionine labelling at which time these molecules migrate to MIIC compartments after synthesis in ER. This result is consistent with the finding that H2-M was co-precipitated with Ii from pH7 to 5 (see Fig. 4.5) which corresponds to the pH gradient between the ER and MIIC.

5.3. Intracellular H2-M/I-A^d association by re-immunoprecipitation

To investigate the association of H2-M with I-A^d in the endocytic pathway, pulse-chase, re-immunoprecipitation and Western analysis have been executed essentially as described in Section 5.2. For the re-immunoprecipitation experiments, the antibody MKD.6 (see Table 2.2.1 for the antibody concentration) was used to re-precipitate I-A^d from anti-H2-M primary precipitates. The membrane was probed with bio-M5/114 for the identification of I-A^d in the anti-I-A^d re-immunoprecipitates. The membrane was stripped for 30 minutes at 50°C in the stripping buffer (see Section 5.2) and blotted again with antibody #104 to detect the presence of H2-M in the same anti-I-A^d re-precipitates.

In this experiment, a protein which gave a band at the size of I-A^d dimer was re-precipitated from the anti-H2-M primary precipitates at all chase times (Fig. 5.4a) and this band displayed as I-A^d immunoreactivity (Fig. 5.4b second lane) by

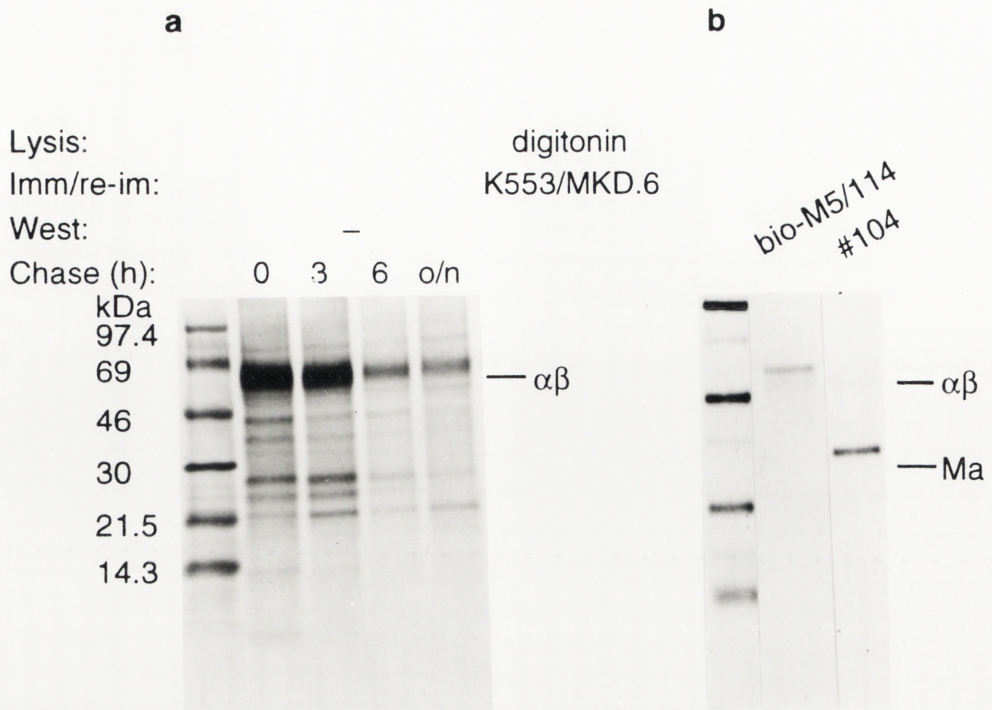


Fig. 5.4. I-A^d was re-precipitated from anti-H2-M immunoprecipitates.

Immunoprecipitation and re-immunoprecipitation methods are basically the same as in the Fig. 5.1 legend except that MKD.6 (anti-I-A^{d}β antibody) was used for the re-immunoprecipitation of I-A^d and associated molecules from anti-H2-M precipitates (**a**). The membrane was immunoblotted with M5/114 for the identification of I-A^d (**b**, second lane) and then immunoblotted again with #104 for the identification of Ma (**b**, third lane) after stripping (see Section 5.3 for details).

Abbreviations: Imm, immunoprecipitation: re-im, re-immunoprecipitation: West, Western blot: o/n, overnight: α, I-A^{d}α: β, I-A^{d}β.

Western analysis. As observed in the course of the pH dependency experiments (see Fig. 4.6), H2-M-associated I-A^d dimers in the re-precipitation with MKD.6 were resistant to the denaturing conditions of sample elution. On the membrane blotted with #104, Ma was detected clearly (Fig. 5.4b third lane) although there was no visible band at the size of Ma on the autoradiography film (Fig.5.4a), presumably due to the difference in relative sensitivity of the two techniques.

Perusal of Fig. 5.4a reveals a distinct band at around 27kDa at 0h chase which appeared to be subject to degradation after the longer chase periods. These bands (25kDa and 22kDa) are likely to be Ii fragments which were intracellularly degraded by proteases (p25 and LIP) but unfortunately they were not identified by immunoanalysis because of inability of the antibody In-1 to recognize the Ii fragments. A band at 31kDa which is visible faintly at 0 and 3h is likely to be Ii-31.

5.4. Discussion

In the re-immunoprecipitation of Ii from the anti-H2-M primary precipitates, Ii-31 was co-precipitated with H2-M in digitonin lysis immediately after pulse (0h, see Fig. 5.1a). This association was sufficiently stable not to be dissociated in the strong detergent, NP40 (see Fig. 5.3). A 28kDa protein in the anti-Ii re-precipitates of digitonin lysis (see Fig. 5.1) may be an Ii fragment which associates with H2-M weakly in the endocytic pathway, because this band originated from Ii-31 after the 3h chase period and it was not seen in the P4H5 re-precipitates from NP40 lysis (see Fig. 5.3). In the reciprocal experiment, H2-M was re-precipitated from anti-Ii primary precipitates until 3h chase or later (see Fig. 5.2a), indicating that the H2-M/Ii association persists in the endocytic pathway for a substantial period after synthesis in the ER. The Ii immunoreactivity associated with H2-M in the endocytic pathway may derive from Ii fragments cleaved by proteases resident in the endosomal compartments. This is evident from the appearance of a band at

28kDa at 3h chase period correlates with the disappearance of Ii-31 in Fig. 5.1a. However, the H2-M-associated Ii fragments were not identified by Western analysis using the anti-Ii antibody, In-1, which recognizes the amino acid residues 2-17 of Ii. In-1 identified only Ii-31 and $(Ii\text{-}31)_2$ throughout these experiments (see Fig. 6.2a). For the identification of these bands by Western analysis, use of an alternative anti-Ii antibody which recognized the CLIP region would be appropriate.

Re-precipitation of I-A^d dimers from anti-H2-M primary precipitates (Fig. 5.4) demonstrated co-precipitation of I-A^d with H2-M immediately after the [^{35}S]-methionine pulse which persisted in decreasing abundance throughout the chase period (see Fig. 5.4a), suggesting that H2-M/I-A^d association occurs not only in MIIC, but also throughout the endocytic pathway, including the ER. This result confirmed those of Pierre *et al.* (1996), who found that DM in HLA-DMA/DMB transfected A20 cells is not restricted only to the MIIC but is found all through the endocytic pathway. By analogy, the I-A^d which was co-precipitated with H2-M after 6h or overnight chase originates from a sub-population which remains in the intracellular compartments, since DM was not found at the cell surface (Pierre *et al.*, 1996)

Dominant bands at 27kDa, 25kDa and 22kDa in the anti-I-A^d re-precipitation from anti-H2-M primary precipitates (Fig. 5.4a) are considered to be Ii fragments, since the intensity of these bands was greatest at the 3h chase period, which is the required time for a protein to migrate to the endosomal compartments in the pulse-chase experiment. Proteins of similar size were precipitated maximally over a range of pH corresponding to that between endosomes and lysosomes (pH6 to pH4) in the anti-I-A^d, anti-Ii and anti-H2-M immunoprecipitations (Fig. 3.5a, 3.5b and 3.5c). Co-precipitation of these Ii fragments, which have been cleaved by proteases in the endosomal compartments, provides good evidence of association

of the three proteins, I-A^d, Ii and H2-M, throughout the endocytic pathway of I-A^d class II antigen presentation.

Overall, it has been demonstrated that H2-M was re-precipitated with Ii until at least 6h after synthesis (Fig. 5.2), I-A^d was re-precipitated with H2-M at all chase times (Fig. 5.4) and either Ii-31 or Ii fragments (LIP, SLIP) were co-precipitated with I-A^d at 0h or 2h chase, respectively (Fig.3.1a). These results suggest that I-A^d class II molecules may associate in a variety of complexes with Ii and H2-M not only immediately after synthesis in the ER but also during transport in the endocytic pathway. Additionally, re-immunoprecipitations of Ii from anti-H2-M precipitates (see Fig. 5.1a & 5.3a) and H2-M from anti-Ii precipitates (Fig. 5.2a) showed bands between 55 to 60kDa at all chase times. These bands in the re-precipitates have not been identified directly, however, bands of the same size have been identified as being I-$A^d\alpha\beta$ heterodimers by Western analysis in the re-immunoprecipitation of I-A^d from the anti-H2-M primary precipitates (Fig. 5.4b). These observations provide further evidence that I-A^d dimers associate with Ii and H2-M throughout the endocytic pathway.

The association of HLA-DM with HLA-DR has been shown to be a direct association at pH5, the pH of MIIC, by Sanderson *et al.* (1996). From the experiments reported here, it is postulated that H2-M is associated with Ii in the ER and Ii fragments in the endocytic pathway (Fig. 5.1, 5.2 and 5.3) and, additionally, it is associated with I-A^d throughout the class II pathway (Fig. 5.4). Then, the question as to how the formation of a physical association of H2-M and Ii arises. It may be a direct association, independent of the involvement of other molecules or an indirect association mediated through other molecules, such as I-A^d class II molecules. To observe the formation of this association in the absence of I-A^d, experiments for the co-precipitation of H2-M or Ii with Ii or H2-M in I-A^d

deficient cells were performed. These experiments are presented in the subsequent chapter.

Chapter 6

6. Characterization of H2-M/Ii association in the absence of I-A^d

6.1. Introduction

The results presented in the preceding Chapters showed that Ii-31 may be co-precipitated with H2-M from A20 cells early in chase period (see Fig.5.1 and Section 5.2 for details) and H2-M was co-precipitated with anti-Ii immunoreactivity for at least 3h into the chase period or later (see Fig. 5.2 and Section 5.2 for details). These results indicated that an H2-M/Ii-31 association occurs in the ER and the association of H2-M and Ii fragments persists along in the endocytic pathway. Previously, Ii fragments, such as LIP and SLIP, have been co-purified with HLA-DM in purified HLA-DR from leupeptin treated .114 cells (Schafer *et al.*, 1996). As discussed in Section 5.4, MHC class II molecules associate with both Ii and Ii fragments, such as LIP and SLIP, throughout the class II pathway (Riberdy *et al.*, 1994). Thus, the question arises as to how the formation of the H2-M/Ii association arises; is H2-M associated with Ii directly in cells or does it require the presence of MHC class II molecules to mediate the interaction?

To address these questions, an I-A^d-deleted cell line, M12.C3, was used to characterize H2-M/Ii association in the absence of I-A^d class II molecules. M12.C3 cells have been generated from the I-A^d expressing B lymphoma cell line, M12, by repeated cycles of negative immunoselection after mutagenesis with γ–irradiation. As a means of verifying the lack of I-A^d expression in these cells, FACS analysis was performed which showed a loss of functional I-A^d expression on the cell surface (Glimcher *et al.*, 1985).

Prior to the investigation of the H2-M/Ii association in M12.C3 cells, the absence of I-A^d class II molecules was confirmed by FACS analysis after staining

with an antibody which binds to cell surface I-A^d. Also, the presence of intracellular Ii and H2-M in the cells was checked by Western analysis of cell lysates. The association of Ii and H2-M in M12.C3 cells was investigated by probing for Ma in anti-Ii immunoprecipitates.

6.2. Comparative expression of I-A^d, Ii and H2-M in A20 cells and M12.C3 cells

6.2.1. Confirmation of cell surface I-A^d absence in M12.C3 cells

The deletion of cell surface expression of I-A^d in M12.C3 cells was verified by FACS analysis and A20 cells were used as a positive control for I-A^d expression. Details for the procedure to identify cell surface expression of I-A^d in M12.C3 and A20 cells have been described in Section 2.7.1. In brief, M12.C3 and A20 cells (20,000 cells) were washed once in FACS medium (1% BSA and 0.05% sodium azide in PBS) and cell surface I-A^d was visualized with the FITC-conjugated anti-I-$A^d\beta$ antibody, M5/114 (FITC-M5/114). Stained cells were washed again to remove the unbound antibody thoroughly and any dead cells were stained with PI before analyzing by flow cytometry. For the determination of the antibody-bound cell surface I-A^d molecules, dead cells were excluded from the analysis. The same process has been followed except without staining the cells with FITC-M5/114 for a negative control of the antibody.

As Fig. 6.1 shows, there were clear differences in the fluorescence intensity between the two cell lines. A20 cells showed a high intensity of fluorescence, which indicates high expression of I-A^d. In contrast, M12.C3 cells showed very low fluorescence intensity compared to A20 cells, which confirmed that cell surface I-A^d expression in M12.C3 cells is relatively very low. Attempts were made to minimize the level of background by more washes and using lower concentration of the antibody. Despite numerous attempts, the fluorescence

Relative expression of I-A^d, Ii and H2-M in A20 and, an I-A^d deletion-mutant, M12.C3 cell lines.

Fig. 6.1. Expression of cell surface I-A^d in A20 and M12.C3 cells.

A20 cells or M12.C3 cells were washed and stained with an antibody reactive to I-A^d (FITC-M5/114). Cells were washed again and incubated with PI to stain dead cells. Cell surface I-A^d on viable cells was analyzed by flow cytometry.

Fig. 6.2. Expression of Ii and H2-M in A20 and M12.C3 cells.

A20 and M12.C3 cells were lysed in 1% NP40 lysis buffer pH7 for 20 min at 4°C. Lysates of both cells were divided into 2 equal portions and one portion of the lysates was non-reduced and non-denatured, the other portion of the lysates was reduced and denatured by boiling for 5 min in the presence of 2.5% 2-ME. Both non-denatured and denatured cell lysates containing 1% SDS were divided again into 2 portions before analysis by 10-20% Tris-Tricine gradient gel electrophoresis followed by immunoblotting with In-1 (**a**) or #104 (**b**).
Abbreviations: M, M12.C3 cells: A, A20 cells: +, denatured: -, non-denatured: 2Ii, $(Ii\text{-}31)_2$.

Fig.6.1

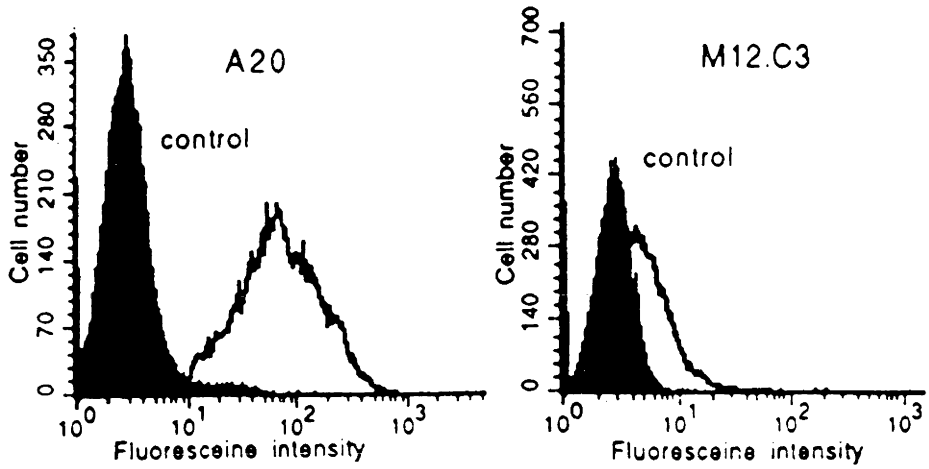
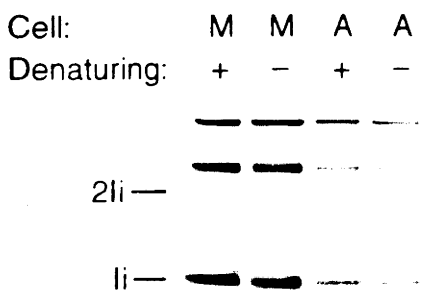
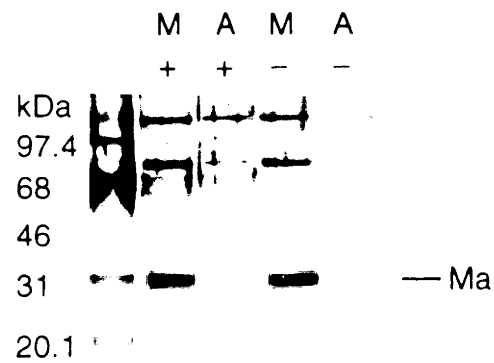


Fig. 6.2a



b



intensity in the sample of M12.C3 cells could not be reduced to exactly the same level as the negative control. However, Glimcher *et al.* (1985) have shown that cell surface I-A^d expression in M12.C3 cells was completely abrogated. In their cytometric analysis using the antibody 34-5-3 (anti-I-A^d), the I-A^d cell surface expression of this cell line (M12.C3) was exactly the same as the negative control. The differences between the result presented here and that obtained by Glimcher *et al.* (1985) may be caused by differences in the non-specific binding properties or cross reactivities of the two antibodies (MKD.6 and 34-5-3) or, alternatively, the cells may now be expressing a low level of cell surface I-A^d after long term maintenance in culture.

6.2.2. Confirmation of the presence of Ii and H2-M in M12.C3 cells

The presence of Ii and H2-M was determined by Western analysis of M12.C3 cell lysates. Lysates from the A20 cell line were used as a positive control for the presence of these molecules. The method of Western analysis for direct identification of Ii or H2-M in both cell lysates has been described in Section 2.5. Briefly, cells were washed once in PBS and lysed in 1% NP40 lysis buffer at pH7 or pH5. Each cell lysate was divided into two portions. One portion of each cell lysate was analyzed without denaturation and the other portion of the lysate was reduced and denatured by boiling before resolution by 10-20% Tris-Tricine SDS-PAGE. The loading of samples on the gel was done replicatively for the purpose of blotting with different antibodies. The proteins separated by the gel electrophoresis were transferred onto nitrocellulose membranes which were subjected to Western analysis for H2-M or Ii on the membrane. The non-specific binding sites on the membranes were blocked and the membranes were then probed with In-1 or #104 for Ii or H2-M identification, respectively. Washed membranes were incubated again with the pertinent secondary antibody, anti-rat IgG-HRP for

the primary antibody, In-1, or anti-rabbit IgG-HRP for #104, respectively. Membranes were washed again and both proteins were detected by the ECL protein detection system.

In both cell lines, as shown in Fig. 6.2a, Ii-31 dimers and monomers were detected under non-denaturing conditions of cell lysates and only Ii monomers were seen in the denatured samples, presumably since the dimer of Ii was dissociated under denaturing conditions.

The presence of Ma was detected in both M12.C3 and A20 cell lysates under both non-denaturing and denaturing conditions (Fig 6.2b). As observed previously, the mobility of Ii and Ma were very similar on the gel. H2-M dimers were not detected in the cell lysates.

Normal levels of Ii and H2-M expression were confirmed in M12.C3 cells by comparison in A20 cells. The apparent relatively higher concentrations of Ii and H2-M in M12.C3 than in A20 cells might have been caused by using the cells at a different growth stage. The M12.C3 cells were morphologically much healthier than A20 cells under microscopy when they were used.

6.3. Association of H2-M with Ii in the absence of I-A^d class II molecules

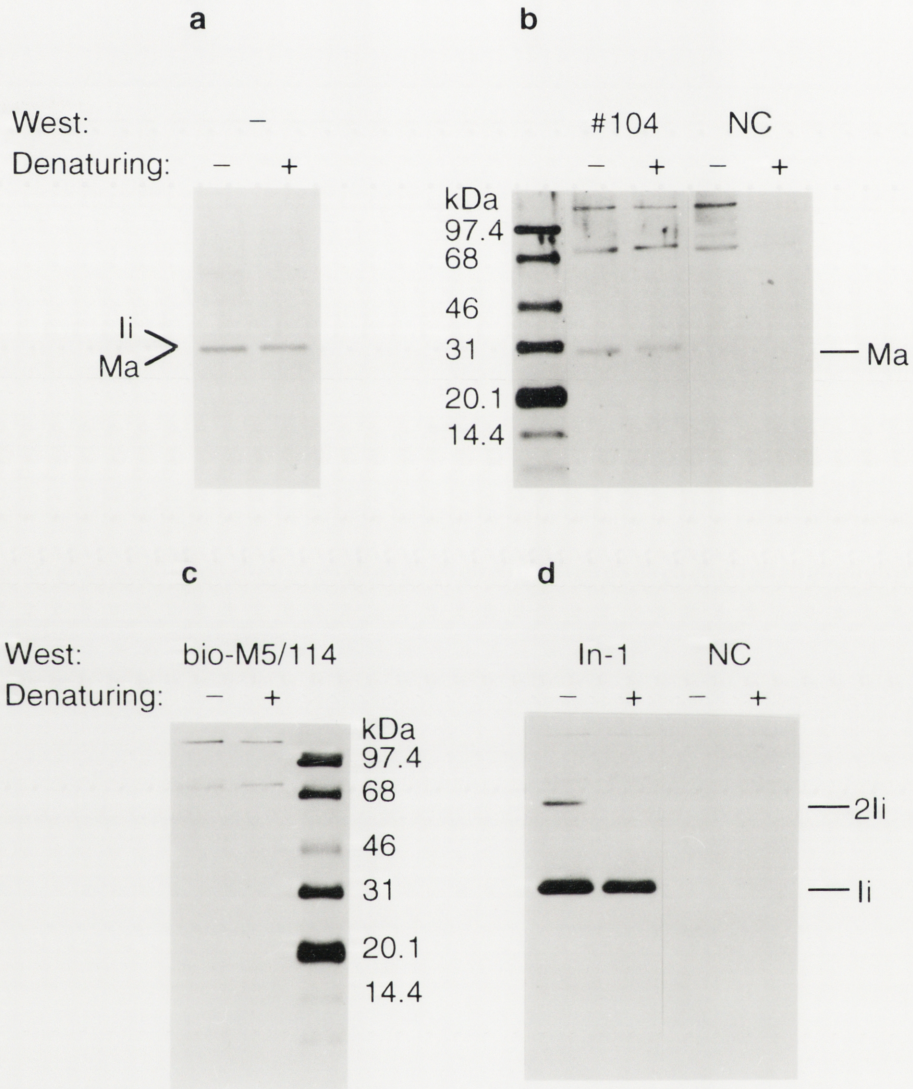
Having established very low levels of expression of I-A^d concomitant with normal to high level expression of H2-M and Ii in the M12.C3 cells, evidence of the association of H2-M with Ii in the absence of I-A^d was investigated by probing for Ma in anti-Ii immunoprecipitates. The immunoprecipitation procedure was essentially the same as that described in Section 3.4, except that 1% NP40 lysis buffer pH7 was used for this immunoprecipitations and samples were analyzed under both non-denaturing and denaturing conditions.

Briefly, M12.C3 cells were depleted of intracellular methionine and labelled with [^{35}S]-methionine for 2h. Cells were then lysed in 1% NP40 lysis buffer at pH7, cell lysates were precleared and Ii and associated molecules were precipitated with P4H5 (anti-Ii). The precipitates were washed in the corresponding lysis buffer and then eluted in Tris-Tricine gel loading buffer under both non-denaturing and denaturing conditions. Eluates were then loaded onto replicate 10-20% Tris-Tricine gradient gels for blotting with various antibodies. Resolved proteins were electrotransferred onto a nitrocellulose membrane followed by exposure to an autoradiography film. The membranes were blotted with bio-M5/114, In-1 or #104 for I-A^d, Ii or H2-M identification, respectively. Secondary antibodies streptavidin-HRP, anti-rat IgG-HRP or anti-rabbit IgG-HRP were used for the primary antibody bio-M5/114, In-1 or #104, respectively.

As shown in Fig. 6.3a, a radioactive band was seen at 31kDa by autoradiography of anti-Ii precipitates from M12.C3 cells under both non-denaturing and denaturing elution conditions. Since Ii-31 and Ma have been found to co-migrate on the gel, Ma identification in these immunoprecipitates was performed by immunoanalysis.

The presence of Ii in M12.C3 cells was verified by detection of Ii-31 dimers and monomers from anti-Ii precipitates by probing with the antibody In-1 (Fig. 6.3d). In Fig. 6.3c, the absence of both I-A^d dimers and monomers from anti-Ii precipitates of M12.C3 cells under both non-denaturing and denaturing conditions was further evidence of lack of I-A^d expression in these cells. Significantly, both I-A^d dimers and monomers have been co-precipitated with Ii in the I-A^d positive A20 cells under these conditions (see Fig. 3.1b).

A 31kDa band which was immunoreactive to #104 from anti-Ii precipitates of M12. C3 cell lysates (Fig. 6.3b) showed that H2-M co-precipitated with Ii in the


a
b
West:
Denaturing:
–
+
Ii
Ma
#104
NC
kDa
97.4
68
46
31
20.1
14.4
Ma
c
d
West:
bio-M5/114
In-1
NC
2Ii
Ii

absence of I-A^d. This result indicates that Ma associates directly with Ii, independent of the presence of I-A^d. Furthermore, the association of these molecules (Ii and H2-M) was sufficiently stable such that H2-M co-precipitated with Ii in NP40 lysis of M12.C3 cells, replicating their co-precipitation from lysates of A20 cells in the same detergent (see Fig. 5.3).

6.4. Ii degradation in I-A^d deficient M12.C3 cells

The observation of a stable, direct association of H2-M with Ii in the absence of I-A^d at pH7 (as shown above) prompted an examination of the degradation of Ii-31 in the endocytic pathway in the absence of I-A^d. Retarded degradation of Ii in anti-DR immunoprecipitates from DM-deleted T2.DR3 cells (Riberdy *et al.*, 1994) has led to the proposal that DM catalyzes Ii proteolysis. The rate of degradation of Ii in the absence of I-A^d was observed by comparing the amount of Ii-31 precipitated at different chase times between M12.C3 and A20 cells which had been pulsed with [^{35}S]-methionine.

Immunoprecipitation of Ii from A20 and M12.C3 cells in a pulse-chase experiment was performed essentially in the same way as described for the immunoprecipitation of Ii at different chase times in Section 3.3, except that 1% NP40 was used to lyse the cells, the chase times were different (0, 2, 4 & 6h) and the eluates were analyzed under denaturing conditions only in this experiment. Briefly, both cell lines were treated identically, being washed and depleted of intracellular methionine for two 30 minutes periods. Cells were then pulsed with [^{35}S]-methionine for 30 min and chased with a ten-fold excess of [^{32}S]-methionine for various time periods (0, 2, 4 or 6h). At the end of the chase period, the cells were washed in chilled PBS and lysed in 1% NP40 lysis buffer at pH7. After preclearing the cell lysates, Ii was immunoprecipitated with P4H5 in the presence of protein A sepharose-6MB. Precipitates were washed in the corresponding lysis

buffer, then extracted under reducing and denaturing conditions in Tris-Tricine gel loading buffer containing 2-ME prior to analysis by 10-20% Tris-Tricine gel electrophoresis. Proteins resolved by the gel electrophoresis were eletrotransferred onto a nitrocellulose membrane and then exposed to autoradiography film to visualize the pattern of Ii degradation at longer chase times and the relative intensity of Ii between M12.C3 and A20 cells at each chase time was then compared by phosphorimager analysis.

The visual differences of Ii-31 intensity by autoradiography (Fig 6.4) between the two cell lines at longer chase times were confirmed by the phosphorimager analysis (Fig. 6.5) and a numerical analysis of the differences is presented in Table 6.1. As evident in Fig. 6.4 and 6.5, the relative expression of Ii-31 was higher in A20 cells initially but Ii-31 was degraded more rapidly in A20 cells than in M12.C3 cells. This suggests that the presence of I-A^d is required for the normal proteolysis of Ii-31 in the endocytic pathway.

As expected, I-A^d dimers which had been co-precipitated with Ii in A20 cells (Fig 6.4a) were not seen in anti-Ii immunoprecipitates of lysates from M12.C3 cells (Fig 6.4b).

6.5. Discussion

The FACS analysis of the cell surface expression of I-A^d in M12.C3 cells revealed only background levels of immunoreactivity. However, the effective absence of I-A^d class II molecules from the MHC class II-mediated antigen presentation system in this cell line was substantiated in several ways. These included lack of I-A^d immunoreactivity by Western analysis with bio-M5/114 from anti-Ii precipitates (see Fig 6.3c) and absence of I-A^d dimers in the autoradiographic images from the pulse-chase immunoprecipitations of Ii from M12.C3 cells (Fig 6.4b).

Fig. 6.4-5 and Table 6.1. Comparison of Ii degradation patterns in the presence and absence of I-A^d.

A20 and M12.C3 cells (1.5 million cells/well of protein gel) were pulsed with [^{35}S]-methionine for 30 min and chased in different time periods (0, 1, 2, 4 or 6h). Chased cells were lysed in 1% NP40 lysis buffer pH7 containing protease inhibitors and precleared cell lysates were incubated with P4H5 in the presence of protein A sepharose-6MB beads for 2h at 4°C to precipitate Ii. Precipitates were washed and eluted, then analyzed by a 10-20% Tris-Tricine gradient gel electrophoresis under denaturing conditions followed by transfer onto a nitrocellulose membrane. The membrane was exposed to an autoradiography film first (**6.4a** and **6.4b**) and the labelled Ii-31 concentration was then compared visually (**6.5**) and counted (**Table.6.1**) at different chase times by phosphorimager analysis.
Abbreviations: αβ, I-A^dαβ

Fig. 6.4a

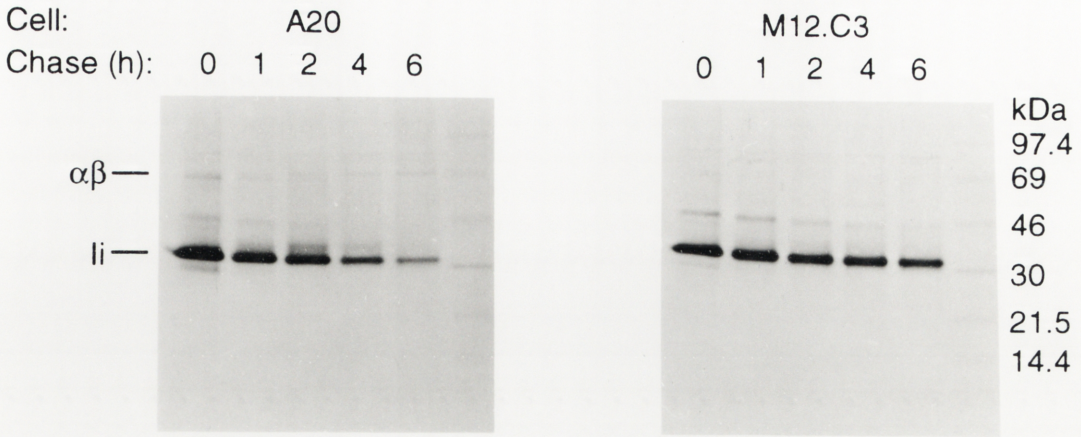


Fig. 6.5

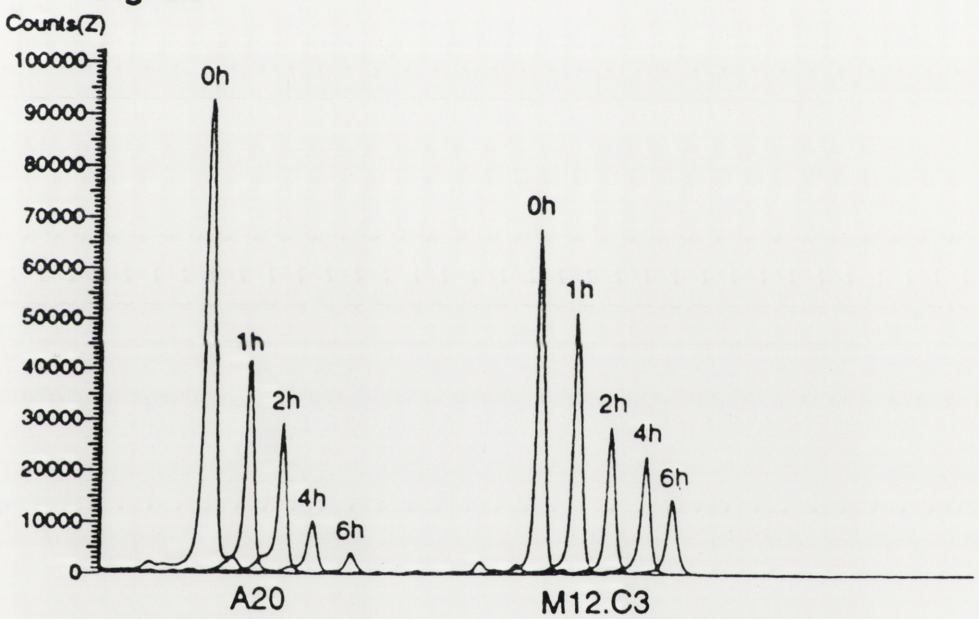


Table 6.1. Phosphorimager ^{35}S count of Ii P31 intensity at different time chases.

Chase (h)	A20	(%)	M12.C3	(%)
0	93,739	(100)	67,682	(100)
1	41,351	(44)	48,777	(72)
2	27,638	(29)	26,456	(39)
4	10,422	(11)	22,734	(33)
6	4,320	(4)	14,342	(21)

The association of H2-M with Ii has been observed to be detergent stable and direct since the co-precipitation occurs in the absence of the I-A^d class II molecules (see Fig. 6.3b). To confirm the direct association of H2-M and Ii in the class II pathway, re-immunoprecipitations of Ii from anti-H2-M or H2-M from anti-Ii precipitates of these cells (M12.C3) might be performed.

The stable, direct association of H2-M with Ii in the class II pathway may be an important functional aspect of the MHC class II-mediated antigen presentation pathway. After synthesis of the three proteins, I-A^d, Ii and H2-M, in the ER, they may be transported together, as complexes, in the endocytic pathway. By the time the final proteolysis of Ii has occurred, leaving CLIP in the complexes, loss of the direct association of Ii-31 with H2-M may allow a conformational change in I-A^d/H2-M complexes in the acidic compartments such that the H2-M catalysed peptide exchange may occur.

Previously, Riberdy *et al.* (1994) have reported that the proteolysis of Ii in DM defective T2.DR3 cells was less efficient than normal and CLIP were generated more slowly than in wild type Pala cells. In this experiment, a reduced rate of degradation of Ii has been observed in I-A^d defective M12.C3 cells relative to wild type A20 cells. Therefore, it appears that both I-A^d and H2-M may be required for efficient proteolytic processing of Ii, perhaps by forming a ternary complex of I-A^d, Ii and H2-M.

Chapter 7

7. Discussion

7.1. General Discussion

In MHC class II antigen presentation in mouse, I-A^d class II molecules require H2-M for the presentation of antigenic peptides to $CD4^+$ T cells (Weenink *et al.*, 1996). The objective of this project was to study the interaction of H2-M with I-A^d and Ii and, hence, to characterize its role in I-A^d class II-mediated antigen presentation. The identification of proteins associated with H2-M was pursued by employing the method of co-immunoprecipitation directed against the three molecules, I-A^d, Ii and H2-M, which were known to have importance for I-A^d class II antigen presentation (Weenink *et al.*, 1996). The conditions of immunoprecipitation were investigated systematically in order to optimize the number and yield of proteins which co-precipitated with molecules involved in the class II pathway.

It is known that HLA-DM and its murine analogue, H2-M, play an important role in MHC class II antigen presentation by assisting the exchange of CLIP for antigenic peptides in the binding groove of MHC class II molecules in antigen processing compartments (Kropshofer *et al.*, 1997). However, the details of the intracellular interactions of this protein and the mechanism of its participation in peptide exchange are still poorly understood. In this study, the former aspect of H2-M biochemistry was explored and there were several novel findings concerning the intracellular association of H2-M with I-A^d and Ii. Hence, the results presented here contribute to a further understanding of the mechanism of MHC class II-mediated antigen presentation.

The immunoprecipitation of proteins in the I-A^d class II pathway was investigated systematically in different solution conditions, such as varying the pH and the chaotropic strength of detergents used for lysis. Protein complexes may

have stabilities and solubilities which vary, depending on the detergents and pH used to lyse cells. This investigation of a range of conditions for immunoprecipitation was critical to achieve the extraction of both the maximum number and the maximum amount of proteins and their intact complexes.

There were significant detergent-dependent differences in the molecules which were co-precipitated. Generally, aggregates or complexes of molecules involved in the class II pathway were precipitated more effectively in the weakly chaotropic detergent, digitonin. By contrast, small proteins appeared to be precipitated more effectively in the strongly chaotropic detergent, NP40. In particular, H2-M-associated molecules were co-precipitated by K553 more effectively in lysis with digitonin rather than NP40 (Fig. 3.4). The most notable example of this detergent-related effect is a protein, p43, which was co-precipitated in digitonin lysates at 0h and after the overnight chase period in the anti-H2-M immunoprecipitates (Fig. 3.4). This suggests that H2-M has a tendency to participate in large, weakly-associated protein complexes and that these intracellular associations may not sufficiently stable to be detected in an immunoprecipitation coupled with lysis in a strong chaotropic detergent. Hence, digitonin was used for cell lysis and immunoprecipitation in experiments designed to co-precipitate molecules which were associated with H2-M.

It was found that MHC class II-associated molecules were co-precipitated differentially in a pH-dependent manner also, which in part depended on the stability of these specific complexes. The floppy form of I-A^d dimers visible at 60kDa were most evident at pH7 but, in contrast, the compact dimeric form of I-A^d at 55kDa showed the greatest abundance at low pH in both the anti-I-A^d and anti-Ii precipitates (Fig. 3.5a and 3.5b). The appearance of the compact form of I-A^d dimers at low pH derived from the population of class II molecules which have bound endogenous peptides in this experiment (see Section 1.3.2 for details). This

observation correlates with the finding of Jensen (1992) that the compact conformation of peptide-loaded I-E^k class II molecules was most stable in SDS at pH5 to 5.5. While Ii-31 and $(Ii\text{-}31)_2$ were co-precipitated with I-A^d class II molecules most abundantly at pH7, the Ii fragments, p25 and p10, were co-precipitated with I-A^d at pH6 only (Fig. 3.5a). These results are taken to reflect the maximal stability of association of these molecules with I-A^d at pH6 or, alternatively, differential selectivity of co-precipitation by MKD.6 at different acidities. Significantly, a small protein band at 3kDa, which corresponds to the size of CLIP, was seen in immunoprecipitates with each antibody at pH5 and 4 (Fig. 3.5a, 3.5b & 3.5c). This observation correlates with the finding of Bangia and Watts (1995) that CLIP binding to I-$E^{d\ \&\ k}$ class II molecules was maximal at pH5.5 in their binding assay. Thus, the use of co-immunoprecipitation to reflect pH-dependence in the stability of I-A^d-CLIP complexes yields a result similar to the binding assay.

Two unidentified proteins were precipitated differentially as a function of pH; a 90kDa protein co-precipitated with both I-A^d and Ii at pH5 only (Fig. 3.5a and 3.5b) and a 43kDa protein was co-precipitated with both of these antibodies most abundantly at pH4 (Fig. 3.5a and 3.5b). These proteins may originate from pH sensitive complexes which are involved in the class II pathway but which have not been detected using the traditional method of immunoprecipitation at pH7. Additionally, an 80kDa protein was co-precipitated with all three molecules, I-A^d, Ii and H2-M, independently of the pH used (Fig. 3.5a, 3.5b & 3.5c). Identification of these molecules, possibly requiring protein sequence analysis, is a prerequisite to establishing a potential role for them in MHC class II-mediated antigen presentation.

The autoradiographs from immunoprecipitation of H2-M and associated molecules by K553 (Fig.3.1c and 3.5c) were characterized by the weak intensity of

specific bands against a high background, particularly at low pH, presumably because this is an unpurified anti-serum and, additionally, H2-M expression in the cell is relatively low (Schafer *et al.*, 1996). Hence, to characterize any difference in the association of H2-M with I-A^d and Ii at different acidity, immunoprecipitations were conducted using digitonin lysis followed by Western analysis. Intact Ii-31 was observed to co-precipitate with H2-M at pH7 only (Fig. 4.4) but H2-M was co-precipitated with Ii immunoreactivity over a range of pH, from 7 to 5 (Fig. 4.5), suggesting that H2-M associates maximally with Ii-31 at pH7 and, apparently, with Ii fragments at pH6 and pH5. The co-immunoprecipitation of H2-M with Ii immunoreactivity at pH7 to 5 allows the possibility that H2-M may associate with Ii-31 after being synthesized in the ER and remains associated with Ii fragments during transport along the endocytic pathway. In the analysis of the I-A^d association with H2-M at different acidities, the result that H2-M and I-A^d were co-immunoprecipitated over the range pH7 to pH4 (Fig. 4.6 & 4.7) demonstrates that this association is sufficiently stable such that it has potential to persist from the ER throughout the endocytic pathway to the MIIC. However, the greatest abundance of I-A^d/H2-M co-precipitation occurred at pH5 in the anti-H2-M immunoprecipitations, inferring maximum stability of this association at this pH. This was not apparent in the anti-I-A^d immunoprecipitations perhaps due to the reduced ability of MKD.6 to precipitate molecules at low pH (Fig. 3.5a) giving rise to a reduction in the apparent difference of the pH-dependence of stability.

The pH dependence of these co-precipitations raises the possibility that H2-M may associate with both Ii-related proteins and I-A^d throughout the class II pathway and that the nature of these associations may be different in different intracellular compartments. In order to investigate these hypotheses, experiments were designed in which [^{35}S]-methionine pulse-chase was coupled with double

immunoprecipitation of these molecules and the precipitates were analyzed by a combination of autoradiography and Western analysis.

The re-immunoprecipitations of Ii and H2-M from the anti-H2-M and anti-Ii primary precipitates, respectively, (Fig. 5.1-5.3) indicated that the H2-M/Ii-31 association persists in the endocytic pathway for a substantial period after synthesis in the ER but that the Ii-immunoreactivity associated with H2-M in the endosomal compartments indeed arises from Ii fragments, as consistent with the results from the pH-dependent co-precipitation experiment. For example, Ii-31 and Ii fragments were co-precipitated with H2-M at pH7 and at pH6 and 5, respectively, in the pH-dependent co-immunoprecipitation experiment (see Fig. 3.5c and Fig. 4.4-5). Also, H2-M and Ii-31 were re-precipitated from anti-Ii and anti-H2-M primary precipitates, respectively, in greatest amounts immediately after pulse with [^{35}S]-methionine (Fig. 5.1-5.3) and Ii fragments were re-precipitated from H2-M primary precipitates at 3 and 6h chases in the pulse-chase experiment (Fig. 5.1a). The re-precipitation of I-A^{d} dimers from anti-H2-M primary precipitates at all chase times (Fig. 5.4) suggests that H2-M/I-A^{d} association occurs not only in the MIIC, but also throughout the endocytic pathway, including the ER. Again, this result is consistent with the results from the pH-dependent immunoprecipitations wherein co-precipitation of these molecules occurred over the pH range 7 to 5, corresponding to the pH gradient between the ER and the MIIC. Taking all these results together, it suggested that I-A^{d} class II molecules may associate in a variety of ternary complexes with Ii and H2-M, not only immediately after synthesis in the ER but also during transport in the endocytic pathway. Consequently, the nature of the formation of the physical association of H2-M and Ii was addressed since the association of HLA-DM with HLA-DR has been shown to be a direct association previously (Sanderson *et al.* 1996).

Analysis of co-immunoprecipitation from an I-A^d-deleted cell line showed that H2-M may associate with Ii-31 in a manner that is both stable and direct and which may occur independently of the presence of I-A^d. This suggests that Ii-31 may function as a chaperone for H2-M folding in the ER and, perhaps, during translocation to the MIIC. Furthermore, a reduced rate of degradation of Ii-31 in the I-A^d-deficient M12.C3 cells relative to wild type A20 cells suggested that the presence of I-A^d may be required for the normal proteolysis of Ii in the endocytic pathway. However, Riberdy *et al.* (1994) have reported previously that DM is required for efficient proteolysis of Ii by showing less efficient degradation of Ii in DM defective T2.DR3 cells than in wild type Pala cells. Therefore, it appears that both I-A^d and H2-M may be required for efficient proteolytic processing of Ii in the endocytic pathway, perhaps, through the formation of a ternary complex of I-A^d, Ii and H2-M.

The relationship between the association of H2-M with Ii and the time post-synthesis was investigated in pulse-chase experiments by double immunoprecipitation. It was found that Ii-31 was re-precipitated from anti-H2-M primary precipitates (Fig. 5.1) immediately after the pulse with [^{35}S]-methionine revealing their association in the ER. Co-immunoprecipitation as a function of pH revealed that Ii-31 and H2-M were co-precipitated at pH7 only (Fig. 4.4), indicating that this interaction is maximally stable at pH7. This is consistent with the finding of Sanderson *et al.* (1996) that Ii-associated DM was detected in the ER of Raji B lymphoma cells after sub-cellular fractionation.

By contrast, H2-M was re-precipitated from anti-Ii primary precipitates until at least 3h or more after the pulse (Fig. 5.2) and it was co-precipitated with Ii-immunoreactivity from pH7 to pH5 in the pH-dependent immunoprecipitation experiments with P4H5 (Fig. 4.5). However, intact Ii was detected from H2-M precipitates immediately after pulse (Fig. 3.1c) or at pH7 (Fig. 4.4) only. These

results indicate a strong possibility that H2-M associates with Ii-related proteins in the endosomal compartments from the ER to MIIC and that the Ii associated with H2-M in the endocytic pathway may be Ii fragments. In spite of attempts by Western analysis with In-1 from anti-H2-M precipitates, the Ii fragments co-precipitated with H2-M were unable to be identified in this experiment, presumably because of the limitations of the low abundance of Ii fragments in intact A20 cells and the sensitivity of Western analysis with the antibody, In-1. However, the Ii fragments, LIP and SLIP, have been shown to be present with DM which co-purified with DR3 from leupeptin-treated .114 cells (Schafer *et al.*, 1996).

Furthermore, Ii-31 was co-precipitated with H2-M from lysates of M12.C3 cells, which lack I-A^d, as well as from lysates of I-A^d-expressing A20 cells in the strong detergent NP40. This suggests that Ii-31 associates with H2-M both in the presence and absence of I-A^d class II molecules and, further, this result indicates that the association of Ii/H2-M may occur directly, being independent of the presence of I-A^d. By contrast, Schafer *et al.* (1996) have reported that LIP and SLIP were co-precipitated in the presence of both DM and DR3 in leupeptin treated .114 cells but not in the absence of either molecule, indicating that their association requires a ternary complex consisting of DR3, Ii and DM. The discrepancy in the requirement for MHC class II molecules to be present for the H2-M/Ii association to occur in these experiments and for the analogous DM/Ii association in the investigations of Schafer *et al.* (1996) may relate to the different species or the different haplotype of MHC class II molecules used.

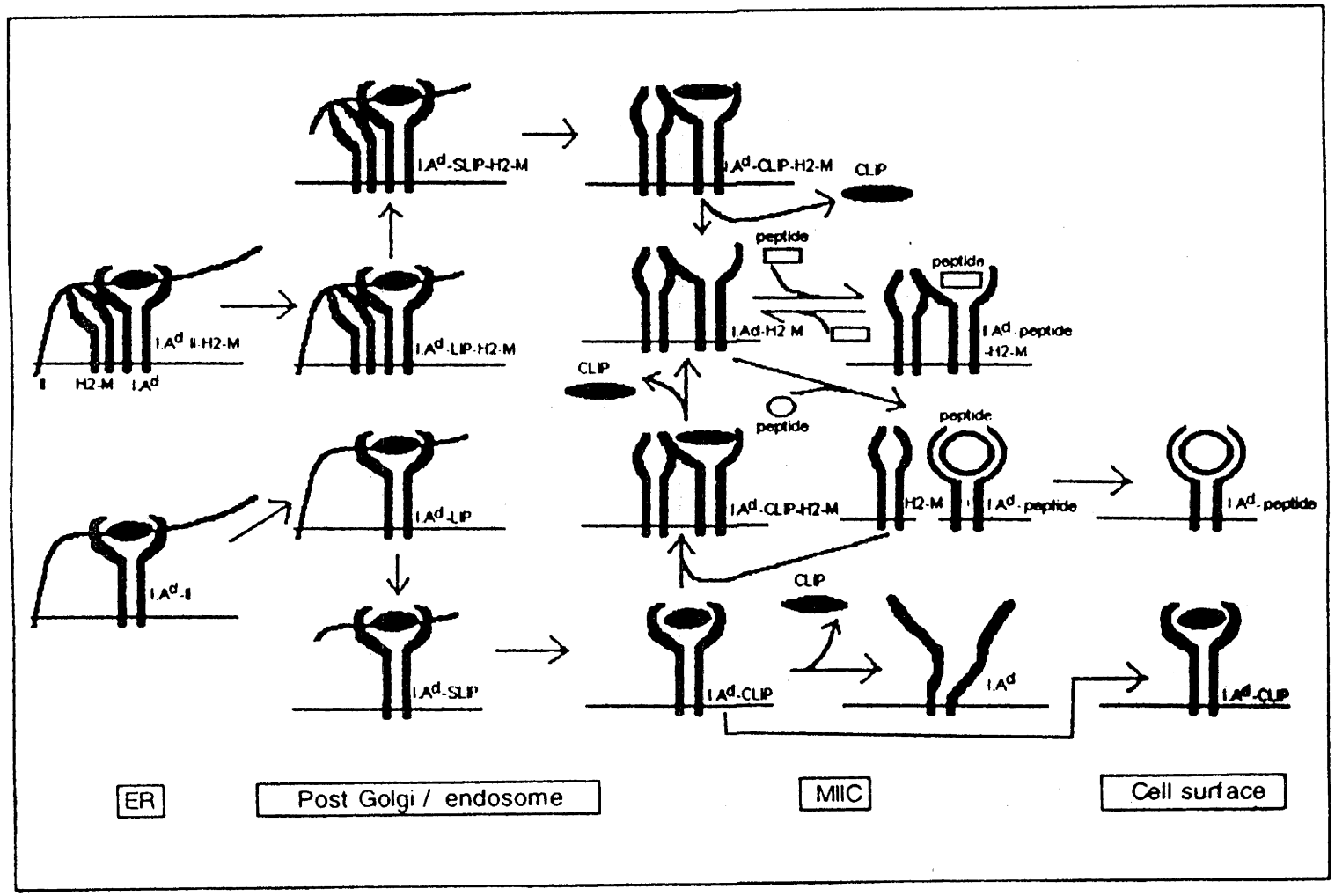
A number of recent reports have addressed DM/DR interactions in the class II pathway. Sanderson *et al.* (1996) have reported that the DM/DR association in Raji cells was observed to be favoured at a low pH (pH5) by immunoprecipitation. However, H2-M/I-A^d co-precipitation from A20 cells was detected over the range pH7 to pH4 (see Fig. 4.6 and 4.7). Additionally, H2-M and I-A^d were re-

precipitated from anti-I-A^d and anti-H2-M primary precipitates, respectively, at all chase times in the pulse-chase experiment (see Fig. 5.4 and 5.5), indicating that H2-M may associate with I-A^d throughout the class II pathway, from the ER, immediately after synthesis, to the MIIC. This result may not be totally unexpected since Denzin *et al.* (1996) have shown that DM associates with class II/Ii complexes in the post-Golgi compartments of Pala cells during degradation of the Ii. Also, Pierre *et al.* (1996) have reported that DM is not restricted to the MIIC. It was found through cell fractionation that DM was present not only in the MIIC but also throughout the endocytic pathway in HLA-DMA/DMB transfected A20 cells.

The observation of the H2-M/Ii and the H2-M/I-A^d associations enabled the proposal of a model for the interaction of H2-M with Ii and I-A^d in the I-A^d class II pathway, as presented in Fig. 7. Alternative to I-A^d-Ii association in the ER as shown in Fig. 1.4, all three molecules, I-A^d, Ii and H2-M, may associate together immediately after synthesis in this compartment, forming a ternary I-A^d/Ii/H2-M complex. This is supported by the co-precipitation of H2-M with Ii and H2-M with I-A^d immediately after [^{35}S]-methionine pulse in A20 cells (see Fig. 5.1 and 5.4) and, also, by previous reports. For example, Roche *et al.* (1991) have demonstrated the association of Ii with DR5 in the ER by sub-cellular fractionation of B-LCL Swei cells and Sanderson *et al.* (1996) have shown Ii association with DM in the ER, also by fractionation of Raji cells. Since DM is expressed to a lower level than DR in .114 cells (1:23 ratio in the cell, Schafer *et al.*, 1996), H2-M is assumed to be synthesized at lower levels than I-A^d class II molecules in A20 cells also. Therefore, H2-M may not participate in all I-A^d/Ii complexes in the ER and while H2-M associates with Ii-31 in the absence of I-A^d it is unlikely that (Ii-H2-M)$_3$ complexes form on statistical grounds. Both H2-M-bound and H2-M-free I-A^d/Ii complexes leave the ER and enter the endocytic

Fig. 7. Schematic model of H2-M interaction in the I-A^d antigen presentation pathway.

I-A^d class II molecules bind to Ii immediately after synthesis in ER to form a nonameric complex, (I-A^d/Ii)$_3$, and H2-M may bind to I-A^d and Ii in this compartment. Ii which has failed to bind to I-A^d may bind also with H2-M in the ER. For I-A^d class II antigen presentation, both I-A^d/Ii/H2-M and I-A^d/Ii complexes leave the ER together and Ii is degraded from the C-terminal region in post-Golgi and endosomal compartments, leaving CLIP in the groove of I-A^d. In the MIIC, CLIP is dissociated from I-A^d by the degradation of Ii and the acidic environment. The peptide binding groove of I-A^d molecule is stabilized by the H2-M until a high-affinity cognate peptide binds. In the absence of H2-M, I-A^d may undergo unfolding and form aggregates that lose the peptide binding function. Concomitant with the binding of a high-affinity peptide, I-A^d conformation is changed and H2-M dissociates from the I-A^d/peptide complex. The I-A^d/peptide complex which has been released from H2-M is then transported to the cell surface and the free H2-M in the MIIC is recycled by binding to an I-A^d/CLIP complex. The I-A^d/CLIP complexes which were failed to bind H2-M are transported also to the cell surface.


I.A^d-SLIP-H2-M
I.A^d-CLIP-H2-M
CLIP
peptide
peptide
I.A^d II-H2-M
I.A^d-LIP-H2-M
I.Ad-H2 M
I.A^d peptide -H2-M
II
H2-M
I.A^d
CLIP
peptide
peptide
I.A^d-LIP
I.A^d-CLIP-H2-M
H2-M
I.A^d-peptide
I.A^d-peptide
I.A^d-II
CLIP
I.A^d-SLIP
I.A^d-CLIP
I.A^d
I.A^d-CLIP
ER
Post Golgi / endosome
MIIC
Cell surface

pathway. Alternatively, a minor cohort of H2-M-free I-A^d/Ii complexes may be transported to endosomal compartments *via* the plasma membrane (Roche *et al.*, 1993).

In the post-Golgi and endosomal compartments, Ii is degraded stepwise leaving Ii fragments in the groove of the class II molecules. Experiments in which the protease inhibitor, leupeptin, was used to treat Raji cells showed accumulation of 21kDa (LIP) and 10kDa (SLIP) fragments of Ii in anti-class II antibody immunoprecipitation (Nguyen *et al.*, 1989). In the putative I-A^d/Ii/H2-M complexes, presumably, H2-M still remains associated with the I-A^d/Ii-fragment complex. Overall, this hypothesis was supported by re-precipitation of I-A^d from anti-H2-M primary precipitates throughout the entire chase period (see Fig. 5.4), re-precipitation of H2-M and I-A^d from anti-Ii precipitates after more than 3h post-synthesis (see Fig. 5.2a) and co-precipitation of Ii/Ii-fragments and H2-M with I-A^d throughout the chase period (see Fig. 3.1a). Furthermore, Ii-31 was detected weakly by immunoblotting with In-1 antibody on the same membrane that I-A^d and Ma have already been identified as shown in Fig. 5.4b (data not shown). Although the majority of peptide loading of I-A^d occurs in the MIIC by exchange of CLIP for antigenic peptides catalysed by H2-M (Weenink *et al.*, 1997), some loading of peptides occurs in the endosomal compartments. This was evident from the finding of Krosphofer *et al.* (1996) that the removal of LIP from I-A^d was enhanced by H2-M in I-A^d, Ii and H2-M transfected Ltk^- cells. As the I-A^d/Ii-fragments/H2-M complex enters the late endosomal compartments or MIIC, the Ii components are more degraded and the shortest products or class II-associated Ii fragments (CLIP) may become dissociated generating an I-A^d/H2-M complex. The fact that the association of Ii with H2-M had a shorter lifetime (3-6h, Fig. 5.1-5.3) than the association of I-A^d with H2-M (overnight, Fig. 5.4) and CLIP did not show binding to purified DM at pH5 (Vogt *et al.*, 1996) may

comprise evidence for the dissociation of CLIP from I-A^d/H2-M complex in the MIIC.

The conformation of the I-A^d/H2-M complex in the acidic environment of MIIC may be different from the conformation of ternary complex, I-A^d/Ii/H2-M, in the ER. This hypothesis was supported by the finding of Ullrich *et al.* (1997) that the conformations of both HLA-DM and HLA-DR3 are pH sensitive and that the interaction between purified DM and DR3/CLIP complex at pH5 was 6- to 7-fold higher than at pH7.3 by fluorimetry. By analogy, a pH induced change in the conformation of I-A^d and H2-M may cause a change in the binding affinity of I-A^d class II molecules for CLIP. Therefore, H2-M may elicit CLIP removal from I-A^d class II molecules, by catalyzing conformational change of the I-A^d in the acidic environment of the MIIC after the loss of a direct association with intact Ii. Sherman *et al.* (1996) have reported that the direct association of DM with DR at pH5 catalysed CLIP dissociation from DR class II molecules. Also, it appears that H2-M stabilizes empty class II molecules by direct mutual association (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997). This is probably an important additional chaperone function of H2-M which enhances I-A^d class II antigen presentation because the empty class II molecules are not stable and tend to aggregate (Stern and Wiley, 1992). It is likely that several rounds of low affinity peptide exchange may occur at the binding groove of I-A^d class II molecules until the I-A^d encounters an optimal peptide ligand. In the anti-I-A^d immunoprecipitation, the SDS-stable dimers of I-A^d which indicated peptide bound class II molecules were most abundant at 6h chase (Fig. 3.1a) and DM has been shown to catalyze the release of peptides which have bound to HLA-DR3 but do not have appropriate anchor residues (van Ham *et al.*, 1996; Kropshofer *et al.*, 1996).

Once the I-A^d/peptide complex is stabilized by the occupation of the peptide binding groove by a high affinity peptide, then the conformation of I-A^d may be

altered again. This, cognate-peptide-induced conformation of I-A^d, may then lead to H2-M dissociation from the I-A^d class II molecules in the MIIC. The tightly bound I-A^d/peptide complex then leaves the MIIC for the cell surface to be scrutinized by $CD4^+$ T cells (Janeway Jr. and Travers, 1994). The H2-M which has dissociated from I-A^d class II molecules stays in the MIIC and may be recycled, for example, binding to an I-A^d/CLIP complex. Denzin *et al.* (1994) have reported that DM in Pala cells has a long half-life (24h) but was not found at the cell surface. In the presence of DM at pH5, the rate of peptide loading onto purified DR2a yielded turnover numbers of 3-12 DR2a molecules *per* minute *per* DM molecule depending on the peptide used in the binding assay (Vogt *et al.*, 1996), which may be good evidence of DM recycling for peptide exchange in the MIIC.

Overall, the findings that H2-M was co-precipitated with Ii for more than 3h chase (see Fig. 5.2) and with I-A^d throughout the chase period (see Fig. 5.4), taken together with the known properties of the association of Ii with I-A^d in the class II pathway (Weenink *et al.*, 1996, see Fig. 3.1a), suggest the formation of a ternary complex, I-A^d/Ii/H2-M, early after synthesis of the molecules in ER. It appears that the complex proceeds along the endocytic pathway with concomitant proteolysis of Ii. This was evident from the observation that I-A^d was re-precipitated from anti-H2-M primary precipitates at 3h after a ^{35}S-methionine pulse (Fig. 5.4), corresponding to the time that these molecules take reach to endosomal compartments after synthesis, and that the Ii fragments, LIP and SLIP, were co-precipitated with H2-M after a 2h chase time (Fig. 3.1c). Attempts were made to identify a large (~150kDa) ternary complex in cells by immunoprecipitation of H2-M or Ii in both NP40 and digitonin lysis followed by Western analysis with a relevant antibody. In spite of several attempts, this was not achieved because of the high background at size of the complex in the non-denatured samples.

Given that H2-M forms a direct stable association with Ii and that this chaperone for I-A^d is present in molar excess, by analogy Ii-31 may act as a chaperone for H2-M in the ER and early endocytic pathway.

The apparent co-migration of H2-M with I-A^d throughout the endocytic pathway, suggests that H2-M may have functions additional to that of catalysing peptide exchange in the MIIC. For example, after Ii has lost its endosomal targeting motifs by degradation in endosomal compartments, H2-M may act as a chaperone for I-A^d in the late endosomal compartments. Furthermore, The retarded degradation of Ii observed in the I-A^d deficient cell line, M12.C3, together with a previous report that the degradation of Ii was slow in DM-deficient T2.DR3 cells (Riberdy *et al.*, 1994) suggest that co-operative interaction of H2-M and I-A^d may be required for the efficient proteolysis of Ii in the endocytic pathway.

It has been reported that HLA-DM mutations occur in patients with autoimmune diseases, such as rheumatoid arthritis, Hashimoto's thyroiditis and seronegative spondyloarthropathy (Kim *et al.* 1996). Variants with valine and isoleucine at amino acid positions 144 and 179, respectively, and alanine at 138 or leucine at 139 of DMB were reported, while none of these amino acid variants have been observed in healthy people. It is possible that mutations of DM may alter its associative properties with Ii or with MHC class II molecules, which may lead to abnormal MHC class II antigen presentation, such as premature CLIP exchange for self peptides in compartments other than MIIC where antigenic peptides are present. Hence, self peptide may be presented by MHC class II molecules to $CD4^+$ T cells. Other possibilities which may result in abrogation of thymic selection and/or T cell anergy are that CLIP may not be readily dissociated from the class II molecules and, hence, antigenic peptides are not presented on the cell surface or partly unfolded conformation of MHC class II molecules loaded with peptide may not be recognised appropriately by $CD4^+$ T cells.

Further studies, to confirm the formation of a I-A^d/Ii/H2-M complex in the class II pathway and the molar ratio of these molecules in the complex are required in order to contribute to the understanding of the complete role of H2-M in I-A^d class II antigen presentation.

7.2. Conclusion

This thesis has presented several novel findings concerning the association of H2-M with Ii and I-A^d in mouse B cell lines.

The results presented here suggest that :

1) There are qualitative and quantitative differences in the molecules immunoprecipitated when different strength detergents are used to lyse cells and precipitate molecules from the I-A^d antigen presentation system. Especially, the association of H2-M with other molecules is weak compared to that of Ii or I-A^d class II molecules.
2) Molecular complexes involved in the MHC class II antigen presentation may be immunoprecipitated in a pH-dependent manner and the complexes appeared to be maximally stable at a pH which reflects their intracellular origin.
3) The functions of H2-M may not be restricted to specialized MIIC only, where MHC class II molecule/CLIP complexes are accumulated, but may be continuous throughout the endocytic pathway.
4) H2-M associates with intact Ii-31 immediately after it is synthesized in the ER and it associates with Ii intermediates in the endocytic pathway.
5) The association of H2-M with I-A^d class II molecules was found throughout the class II pathway from immediately after its synthesis in the ER.
6) The glycoproteins, I-A^d, Ii and H2-M, may form a membrane-associated ternary complex, I-A^d/Ii/H2-M, early after synthesis in the ER, which is transported in the endocytic pathway.

7) H2-M may associate with Ii directly and independently of the presence of I-A^d class II molecules and this association is of sufficient strength not to be dissociated in the chaotropic detergent, NP40, at pH7.

8) In I-A^d deficient cells, the degradation of Ii was retarded as has been shown previously for HLA-DM deficient cells. Therefore, co-operative interaction of H2-M and I-A^d may be required for efficient proteolytic processing of Ii in the endocytic pathway.

9) Given the stability of the direct interaction of H2-M with Ii at pH7, Ii may serve to chaperone H2-M in the early stages of the endocytic pathway. In turn, the stable association of H2-M with I-A^d throughout the class II pathway indicates that H2-M itself may chaperone I-A^d after Ii is degraded in the endocytic pathway.

10) The presence of a ternary complex, I-A^d/Ii/H2-M, was not identified by double-immunoprecipitation followed by immunoblotting and, hence, further experiments, such as single particle analysis by electron microscopy, may be required to confirm the existence of this complex and to determine its stoichiometry.

References

Bakke, O. and Dobberstein, B. (1990). MHC Class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell. **63**:707-716.*

Bangia, N. and Watts, T. (1995). Evidence for invariant chain 85-101 (CLIP) binding in the antigen binding site of MHC class II molecules. *International imm. 7:1585-1591.*

Bikoff, E. K., Germain, R. N. and Robertson, E. J. (1995). Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Imm. 2:301-310.*

Bjorkman, A. J. and Mowbray, S. L. (1998). Multiple open forms of ribosome-binding protein trace the path of its conformational change. *J. Mol. Biol. **279(3)**:651-664.*

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett W. S., Strominger, J. L. and Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature. **329(6139)**:506-512.*

Blum, J. S. and Cresswell, P. (1988). Role for intracellular proteases in the processing and transport of class II HLA antigens. *Imm. **85**:3975-3979.*

Braciale, T. J. (1992). Antigen processing for presentation by MHC class I molecules. *Current Opin. in Imm. 4:59-62.*

Brooks, A. G., Campbell, P. L., Reynolds, P., Gautam, A. M. and McCluskey, J. (1994). Antigen presentation and assembly by mouse I-A^k class II molecules in human APC containing deleted or mutated HLA DM genes. *The Journal of Imm.* ***153****:5382-5392.*

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature.* ***364:****33-39.*

Busch, R., Cloutier, I., Sekaly, R-P. and Hammerling, G. J. (1996). Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *The EMBO Journal.* ***15:2:****418-428.*

Campbell, D. J. and Shastri, N. (1998). Bacterial surface proteins recognized by $CD4^+$ T cells during murine infection with Listeria monocytogenes. *J. Immunol.* ***161(5):****2339-2347.*

Cho., S., Attaya, M., Brown, M. G. and Monaco, J. J. (1991). A cluster of transcribed sequences between the P*b* and O*b* genes of the murine major histocompatibility complex. *Imm.* ***88:****5197-5201.*

Cho, S., Attaya, M. and Monaco, J. J. (1991'). New class II-like genes in the murine MHC. *Nature.* ***353:****573-576.*

Copier, J., Kelijmeer, M. J., Ponnambalam, S., Oorschot, V., Potter, P., Trowsdale, J and Kelly, A. (1996). Targeting signal and subcellular compartments

involved in the intracellular trafficking of HLA-DMB. *The Journal of Imm.* ***157****:1017-1027.*

Cresswell, P. (1992). Chemistry and functional role of the invariant chain. *Current Opinion in Imm.* ***4:*** *87-92.*

Cresswell, P. (1994). Getting peptides into MHC class II molecules. *Current Biology.* ***4:6****541-543.*

Cresswell, P. (1996). Invariant chain structure and MHC class II function. *Cell.* ***84****:505-507.*

Denzin, L. K. and Cresswell, P. (1995). HLA-DM induces CLIP dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell.* ***82****:155-165.*

Denzin, L. K., Hammond, C. and Cresswell, P. (1996). HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J. Exp. Med.* ***184****:2153-2165.*

Denzin, L. K., Robbins, N. F., Carboy-Newcomb, C. and Cresswell, P. (1994). Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity.* ***1****:595-605.*

Engelhard, V. H. (1994). How cells process antigens. *Scientific American.* ***August****:44-51.*

Fairchild, P. J. (1998). Presentation of antigenic peptides by products of the major histocompatibility complex. *J. Pept. Sci.* ***4(3):182-194.***

Gautam, A. M., Pearson, C., Quinn, V., McDevitt, H. O. and Milburn, P. J. (1995). Binding of an invariant-chain peptide, CLIP, to I-A major histocompatibility complex class II molecules. *Imm.* ***92:335-339.***

Germain, R. N., Hendrix, L. R. (1991). MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature.* ***353:134-139.***

Ghosh, P., Amaya, M., Mellins, E. and Wiley, D. C. (1995). The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature.*

Glimcher, L. H., Mckean, D. J., Choi, E. and Seidman, J. G. (1985). Complex regulation of class II gene expression : analysis with class II mutant cell lines. *The journal of Imm.* ***135:*** *3542-3550.*

Green, J. M., DeMars, R., Xu, X. and Pierce, S. K. (1995). The intracellular transport of MHC class II molecules in the absence of HLA-DM. *The Journal of Imm.* ***155:****3759-3768.*

Harding, C. V. and Geuze, H. J. (1992). Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. *The Journal of Cell Biol.* ***119:3****:531-542.*

Harding, C. V., Roof, R. W., Allen, P. M. and Unanue, E. R. (1991). Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Imm. **88**:2740-2744.*

Haracska, L. and Udvardy, A. (1997). Mapping the ubiquitin-binding domains in the p54 regulatory complex subunit of the Drosophila 26S protease. *FEBS Lett. **412(2)**:331-336.*

Hughes, E. A. and Cresswell, P. (1998). The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. *Curr. Biol. **8(12)**:709-712.*

Ingvarsson, S., Lagerkvist, A. C., Martensson, C., Granberg, U., Ifversen, P., Borrebaeck, C. A. and Carlsson, R. (1995). Antigen-specific activation of B cells *in vitro* after recruitment of T cell help with superantigen. *Immunotechnology. **1(1)**:29-30.*

Janeway Jr., C. A. and Travers, P. (1994). Immunobiology: The immune system in health and disease. *Blackwell Scientific Publications. New York and London. 4:1-4:30 and 7:16-7:19pp.*

Jensen, P. E. (1992). Long-lived complexes between peptide and class II major histocompatibility complex are formed at low pH with no requirement for pH neutralization. *J. Exp. Med. **176**:793-798.*

Jones, P. P., Murphy, D. B., Hewgill, D. and McDevitt, H. O. (1978). Detection of a common polypeptide chain in I-A and I-E sub-region immunoprecipitates. *Immunochemistry.* ***16****:51-60.*

Karlsson, L., Peleraux, A., Lindstedt, R., Lijedahl, M. and Per Peterson, A. (1994). Reconstitution of an operational MHC class II compartment in non-antigen-presenting cells. *Sci.* ***266****:1569*-1573.

Kelly, A. P., Monaco, J. J., Cho, S. and Trowsdale, J. (1991). A new human HLA class II-related locus, DM. *Nature.* ***353****:571-572.*

Kim, T. G., Carrington, M., Choi, H. B., Kim, H. Y. and Han, H. (1996). Three HLA-DMB variants in Korean patients with autoimmune diseases. *Human immunology.* ***46****:58-60.*

Kropshofer, H., Arndt, S. O., Moldenhauer, G., Hammerling, G. J. and Vogt, A. B. (1997). HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. *Imm.* ***6****:293-302.*

Kropshofer, H., Hammerling, G. J. and Vogt, A. B. (1997). How HLA-DM edits the MHC class II peptide repertoire: survival of the fittest? *Imm. Today.* ***18:2:****77-80.*

Kropshofer, H., Vogt, A. B. and Hammerling, G. J. (1995). Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding. *Imm.* ***92****:8313-8317.*

Kropshofer, H., Vogt, A. B., Moldenhauer, G, Hammer, J., Blum, J. S. and Hammerling, G. J. (1996). Editing of the HLA-DR-peptide repertoire by HLA-DM. *The EMBO Journal.* ***15:22:****6144-6154.*

Kropshofer, H., Vogt, A. B., Stern, L. J. and Hammerling, G. J. (1995). Self release of CLIP in peptide loading of HLA-DR molecules. *Science.* ***270:*** *1357-1359.*

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* ***227:****680-685.*

Lamb, C. A. and Cresswell, P. (1992). Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *The Journal of Imm.* ***148****:3478-3482.*

Liljedahl, M., Kuwana, T., Fung-Leung, W-P., Jackson, M. R., Per Peterson, A. and Karlsson, L. (1996). HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *The EMBO Journal.* ***15:18:****4817-4824.*

Lindstedt, R., Liljedahl, M., Peleraux, A., Per Peterson, A. and Karlsson, L. (1995). The MHC class II molecule H2-M is targeted to an endosomal compartment by a tyrosine-based targeting motif. *Imm.* ***3:****561-572.*

Madden, D. R., Gorga, J. C., Strominger, J. L. and Wiley, D. C. (1992). The three-dimensional structure of HLA-B27 at 2.1 A resolution suggests a general mechanism for tight peptide binding to MHC. *Cell.* ***70(6):****1035-1048.*

Maffei, A., Papadopoulos, K. and Harris, P. E. (1997). MHC class I antigen processing pathways. *Hum. Immunol.* ***54(2):91-103.***

Maric, M. A., Taylor, M. D. and Blum, J. S. (1994). Endosomal aspartic proteinases are required for invariant-chain processing. *Imm.* ***91:2171-2175.***

Marks, M. S., Blum, J. S., Cresswell, P. (1990). Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigen. *The Journal of Cell Biol.* ***111:839-855.***

Martin, W. D., Hicks, G. G., Mendiratta, S. K., Leva, H. I., Ruley, H. E. and Van Kaer, L. (1996). H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell.* ***84:543-550.***

Mehringer, J. H., Harris, M. R., Kindle, C. S., M^{c}court, D. W. (1991). Characterization of fragments of the murine Ia-associated invariant chain. *The Journal of Imm.* ***146:920-927.***

Mellman, I., Fuchs, R. and Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. *Ann. Rev. Biochem.* ***55:663-700.***

Miyazaki, T., Wolf, P., Tourne, S., Waltzinger, C., Dierich, A., Barois, N., Ploegh, H., Benoist, C. and Mathis, D. (1996). Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell.* ***84:531-541.***

Monaco, J. J. (1992). Genes in the MHC that may affect antigen processing. *Curr. Opin. in Imm.* ***4:70-73.***

Morris, P., Shaman, J. Attaya, M. Amaya, M., Goodman, S., Bergman, C., Monaco, J. J. and Melllins, E. (1994). An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature.* ***368:551-558.***

Murthy, V. L., Stern, L. J. (1997). The class II MHC protein HLA-DR1 in complex with an endogenous peptide: implication for the structural basis of the specificity of peptide binding. *Structure.* ***5(10):1385-1396.***

Naujokas, M. F., Arneson, L. S., Fineschi, B., Peterson, M. E., Sitterding, S., Hammond, A. T., Reilly, C., Lo, D. and Miller, J. (1995). Potent effects of low levels of MHC class II-associated invariant chain on $CD4^+$ T cell development. *Imm.* ***3:359-372.***

Neefjes, J. (1995). Synthesis and assembly of MHC-peptide complexes. *Imm. Today.* ***16:7:359-362.***

Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J. and Ploegh, H. L. (1990). The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell.* ***61:171-183.***

Newcomb, J. R. and Cresswell, P. (1993). Structural analysis of proteolytic products of MHC class II-invariant chain complexes generated in vivo. *The Journal of Imm.* ***151:4153-4163.***

Nguyen, Q. V., Knapp, W. and Humphreys, R. E. (1989). Inhibition by leupeptin and antipain of the intracellular proteolysis of Ii. *Human Imm.* ***24****:153-163.*

Nydam, T., Wade, T. K., Yadati, S., Gabriel, J. L., Barisas, B. G. and Wade, W. F. (1998). Mutations in MHC class II dimer of dimers contact residues: effects on antigen presentation. *Int. Immunol.* ***10(8):****1237-1249.*

O'Sullivan, D. M., Noonan, D. and Quaranta, V. (1987). Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J. Exp. Med.* ***166****:444-460.*

Pepteraux, A., Karlsson, L., Chambers, J. and Per Peterson, A. (1996). Genomic organization of a mouse MHC class II region including the H2-M and Lmp2 loci. *Immunogenetics.* ***43****:204-214.*

Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. and Geuze, H. J. (1991). Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature.* ***349:****669-676.*

Pierre, P., Denzin, L. K., Hammond, C., Drake, J. R., Amigorena, S., Cresswell, P. and Mellman, I. (1996). HLA-DM is localized to conventional and unconventional MHC class II-containing endocytic compartments. *Imm.* ***4****:229-239.*

Pinkoski, M. J., Hobman, M., Heibein, J. A., Tomaselli, K., Li, F., Seth, P., Froelich, C. J. and Bleackley, R. C. (1998). Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood.* ***92(3):****1044-1054.*

Reyes, V. E., Lu, S. and Humphreys, R. E. (1991). Cathepsin B. cleavage of Ii from class II MHC α- and β-chains. *The Journal of Imm.* ***146****:3877-3880.*

Riberdy, J. M., Avva, R. R., Geuze, H. J. and Cresswell, P. (1994). Transport and intracellular distribution of MHC class II molecules and associated invariant chain in normal and antigen-processing mutant cell lines. *The Journal of Cell Biol.* ***125:6:****1225-1237.*

Riberdy, J. M., Newcomb, J. R., Surman, M. J., Barbosa, J. A. and Cresswell, P. (1992). HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature.* ***360****:474-477.*

Riese, R. J., Wolf, R. R., Bromme, D., Natkin, L. R., Villadangos, J. A., Ploegh, H. L. and Chapman, H. A. (1996). Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Imm.* ***4****:357-366.*

Roche, P. A., Marks, M. S. and Cresswell, P. (1991). Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature.* ***354****:392-394.*

Roche, P. A., Teletski, C. L., Stang, E., Bakke, O. and Long, E. O. (1993). Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Cell. Biol.* ***90****:8581-8585.*

Rudensky, A. Y., Maric, M., Eastman, S. Shoemaker, L., DeRoos, P. C. and Blum, J. S. (1994). Intracellular assembly and transport of endogenous peptide-MHC class II complexes. *Imm. **1**:585-594.*

Rudensky, A. Y., Preston-Hurlburt, P., Hong, S-C., Barlow, A. and Janeway Jr., C. A. (1991). Sequence analysis of peptides bound to MHC class II molecules. *Nature. **353**:622-627.*

Sanderson, F., Kleijmeer, M. J., Kelly, A., Verwoerd, D., Tulp, A., Neefjes, J. J., Geuze, H. J. and Trowsdale, J. (1994). Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Sci. **266**:1566-1569.*

Sanderson, F., Powis, S. H., Kelly, A. P. and Trowsdale, J. (1994). Limited polymorphism in HLA-DM does not involve the peptide binding groove. *Immunogenetics. **39**:56-58.*

Sanderson, F., Thomas, C., Neefjes, J. and Trowsdale, J. (1996). Association between HLA-DM and HLA-DR in vivo. *Imm. **4**:87-96.*

Schafer, P H., Green, J. M., Malapati, S., Gu, L. and Pierce, S. K. (1996). HLA-DM is present in one-fifth the amount of HLA-DR in the class II peptide-loading compartment where it associates with leupeptin-induced peptide (LIP)-HLA-DR complexes. *The Journal of Imm. **157**:5487-5495.*

Shachar, I., Elliott, E. A., Chasnoff, B., Grewal, I. S. and Flavell, R. A. (1995). Reconstitution of invariant chain function in transgenic mice in vivo by individual P31 and p41 isoforms. *Imm. **3**:373-383.*

Sherman, M. A., Weber, D. A. and Jensen, P. E. (1996). DM enhances peptide binding to class II MHC release of invariant chain-derived peptide. *Imm. 3:197-206.*

Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E., and Zailer, D. M. (1995). Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature. 375:802-806.*

Smyth, M. J., Krasovskis, E. and Johnston, R. W. (1998). Fas ligand-mediated lysis of self bystander targets by human papillomavirus-specific $CD8^+$ cytotoxic T lymphocytes. *J. Virol. 72(7):5948-5954.*

Stebbins, C. C., Loss Jr., G. E., Elias, E. G., Chervonsky, A. and Sant, A. J. (1995). The requirement for DM in class II-restricted antigen presentation and SDS-stable dimer formation is allele and species dependent. *J. Exp. Med. 181:223-234.*

Stebbins, C. C., Peterson, M. E., Suh, W. M. and Sant, A. J. (1996). DM-mediated release of a naturally occurring invariant chain degradation intermediate from MHC class II molecules. *The Journal of Imm. 157:4892-4898.*

Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C,. Urban, R. G., Strominger, J. L. and Wiley, D. C. (1994). Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature. 368:215-221.*

Stern, L. J., Wiley, D. C. (1992). The human class II MHC protein HLA-DR1 assembles as empty αβ heterodimers in the absence of antigenic peptide. *Cell. **68**:465-477.*

Strubin, M., Berte, C. and Mach, B. (1986). Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *The EMBO Journal. **5:13**:3483-3488.*

Stumptner, P. and Benaroch, P. (1997). Interaction of MHC class II molecules with the invariant chain: role of the invariant chain (81-90) region. *The EMBO Journal. **16:19**:5807-5818.*

Torres, B. A., Tanabe, T. and Johnson, H. M. (1996). Characterization of Nef-induced CD4 T cell proliferation. *Biochem. Biophys. Res. Commun. **225(1)**:54-61.*

Tourne, S., Miyazaki, T., Oxenius, A., Klein, L., Fehr, T., Kyewski, B., Benoist, C. and Mathis, D. (1997). Selection of a broad repertoire of $CD4^+$ T cells in H2-Ma % mice. *Imm. 7:187-195.*

Townsend, K., Sallberg, M., O'Dea, J., Banks, T., Driver, D., Sauter, S., Chang, S. M., Jolly, D. J., Mento, S. J., Millich, D. R. and Lee, W. T. (1997). Characterization of $CD8^+$ cytotoxic T-lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. *J. Virol. **71(5)**:3365-3374.*

Ullrich, H. J., Doring, K., Gruneberg, U., Jahnig, F., Trowsdale, J. and van Ham, S. M. (1997). Interaction between HLA-DM and HLA-DR involves regions that undergo conformational changes at lysosomal pH. *Imm. **94:13163-13168.***

van Ham, S. M., Gruneberg, U., Malcherek, G., Broker, I., Melms, A. and Trowsdale, J. (1996). Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J. Exp. Med. **184:2019-2024.***

Vigna, J. L., Smith, K. D. and Lutz, C. T. (1996). Invariant chain association with MHC class I. *The Journal of Imm. **157:4503-4510.***

Viville, S., Neefjes, J., Lotteau, V., Dierich, A., Lemeur, M., Ploegh, H., Benoist, C. and Mathis, D. (1993). Mice lacking the MHC class II-associated invariant chain. *Cell. **72:635-648.***

Vogt, A. B., Kropshofer, H., Moldenhauer, G. and Hammerling, G. J. (1996). Kinetic analysis of peptide loading onto HLA-DR molecules mediated by HLA-DM. *Imm. **93:9724-9729.***

Warmerdam, P. A. M., Long, E. O. and Roche, R. A. (1996). Isoforms of the invariant chain regulate transport of MHC class II molecules antigen processing compartments. *The Journal of Cell Biol. **133:2:281-291.***

Weenink, S., Averdunk, H., Boston, T., Boswarva, V., Guery, J-C., Adorini, L., Mellins, E., McCluskey, J. and Gautam, A. M. (1996). Impaired antigen

presentation by murine I-A^d class II MHC molecules expressed in normal and HLA-DM-defective human B cell lines. *International Imm.* ***9:6:****101-108.*

Wolf, P. R. and Ploegh, H. L. (1995). How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. *Annu. Rev. Cell Dev.* ***11****:267-306.*

Wong, P. and Rudensky, A. Y. (1996). Phenotype and function of $CD4^+$ T cells in mice lacking invariant chain. *The Journal of Imm.* ***156:6:****2133-2142.*

Wubbolts, R., Fernandez-Borja, M., Oomen, L., Verwoerd, D., Janssen, H., Calafat, J., Tulp, A., Dusseljee, S. and Neefjes, J. (1996). Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *The Journal of Cell Biol.* ***135:3:****611-622.*

Zhong, G., Castellino, F., Romagnoli, P. and Germain, R. N. (1996). Evidence that binding site occupancy is necessary and sufficient for effective major histocompatibility complex (MHC) class II transport through the secretory pathway redefines the primary function of class II-associated invariant chain peptides (CLIP) *The Journal of Experimental Medicine.* ***184:****2061-2066.*